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JA

Anticipated Classification
of this Application:
Class 530 Subclass 402

Prior Application:
Examiner E. Longton
Group Art Unit 1653

Attorney's
Docket
No 0336/25775-CZ-AZ-
JPW/EMW/MMM

January 27, 2000

jc525 U.S. PTO
09/492971
01/27/00

01/27/00
13658 U.S. PTO

HONORABLE ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

S I R:

This is a request for filing a X CONTINUATION
 DIVISIONAL CONTINUATION-IN-PART application under
 X 37 C.F.R. §1.53(b) 37 C.F.R. §1.53(d), of pending prior application
Serial No. 08/909,140 filed on August 11, 1997 of
Tikva Vogel et al. for
Inventor(s)

FIBRIN BINDING DOMAIN POLYPEPTIDES AND USES AND METHODS OF PRODUCING SAME
Title of Invention

1. X Enclosed is a copy of the prior application, as originally
filed and an affidavit or declaration verifying it as a
true copy.
2. A verified statement to establish small entity status under
37 C.F.R. §1.9 and 1.27
 is enclosed.
 was filed in the prior application and such status
is still proper and desired (37 C.F.R. §1.28(a)).
3. X The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE		FEE	
					SMALL ENTITY	OTHER ENTITY	SMALL ENTITY	OTHER ENTITY
Total Claims	9-20	=	0	X	9	18	= \$	\$ 0
Independent Claims	2-3	=	0	X	39	78	= \$	\$ 0
Multiple Dependent Claims Presented: <u> </u> Yes <u> X </u> No					130	260	= \$	\$ 0
BASIC FEE							\$345	\$ 690
TOTAL FEE							\$	\$ 690

*If the difference in Col. 1 is
less than zero, enter "0" in
Col. 2.

filing an application pursuant to this section expressly abandons the
parent application.

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4. X The Commissioner is hereby authorized to charge payment of the following fees associated with this application or credit any overpayment to Deposit Account No. 03-3125.
- X Any additional filing fees required under 37 C.F.R. §1.16.
- X Any patent application processing fees under 37 C.F.R. §1.17.
- The issue fees set forth in 37 C.F.R. §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. §1.311(b).
5. X Three copies of this sheet are enclosed.
6. X A check in the amount of \$ 690.00 is enclosed.
7. Cancel claims .
8. Amend the specification by inserting before the first line the sentence: --This is a continuation division of application Serial No. , filed .--
9. X 62 Sheet(s) of informal X formal drawing(s) is/are enclosed.
10. Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.
11. X Priority of application No. 2,006,929-5 filed on December 29, 1989 in Canada (country) is claimed under 37 U.S.C. §119.
- X The certified copy of the priority application has been filed in prior application Serial No. 08/058,241, filed May 4, 1993.
12. X The prior application is assigned of record to Bio-Technology General Corp..
13. X A preliminary amendment is enclosed.
14. X The power of attorney in the prior application is to:

John P. White, Reg. No. 28,678; Thomas F. Moran, Reg. No. 16,579; Christopher C. Dunham, Reg. No. 22,031; Ivan S. Kavrukov, Reg. No. 25,161; Thomas G. Carulli, Reg. No. 30,616; Robert D. Katz, Reg. No. 30,141; Peter J. Phillips (Reg. No. 29,691; Antoinette F. Konski, Reg. No. P34,202; Catherine R. Roseman, Reg. No. P34,240; and Sarah B. Adriano, Reg. No. P34,470,

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- (a) X The power appears in the original papers in the prior application, and a copy is also enclosed.
- (b) Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
- (c) X Address all future communications to:
(May only be completed by applicant,
or attorney or agent of record.)

John P. White

Cooper & Dunham LLP

1185 Avenue of the Americas

New York, New York 10036

15. X Also enclosed Express Mail Certificate of Mailing No.

EE474773618US dated January 27, 2000.

16. X I hereby verify that the attached papers are a true copy of prior application Serial No. 08/909,140 as originally filed on August 11, 1997.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

January 27, 2000

Date

Signature

John P. White, Reg. No. 28,678

 INVENTOR(S)

 ASSIGNEE OF COMPLETE INTEREST

 X

 ATTORNEY OR AGENT OF RECORD

 FILED UNDER 37 C.F.R. §1.34(a)

Address of Signator:

Cooper & Dunham LLP

1185 Avenue of the Americas

New York, New York 10036

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Serial No. 07/291,951, filed December 29, 1988, now
abandoned.--

Page 11, line 17, delete "(G)" and replace with --(Q)--.

Page 11, line 18, delete "G-A-G-G" and replace with --Q-A-Q-Q--.

Page 12, line 24, delete "This figure shows" and replace with
--All panels (A-H) of this figure show--.

Page 12, line 25, after "nucleotide sequence" insert --(continued
from panel to panel)--.

Page 12, line 28, after "prepared" insert --(Panel A: pairs 1 to
4 and Panel B: pairs 5-7)--.

Page 26, line 17, after "(FN)" insert --(Panel A)--.

Page 26, line 18, after "(31kD)" insert --(Panel B)--.

Page 29, line 6, delete "_____" and replace with --68328--.

Page 29, line 23, delete "975-25" and replace with --197-10--.

Page 41, line 10, delete "_____" and replace with --68328--.

Page 45, line 14, delete "_____" and replace with --68328--.

Page 52, line 23, delete "_____" and replace with --68328--.

Page 52, line 35, delete "_____" and replace with --68328--.

Page 105, line 21, delete "_____" and replace with --68328--.

Page 64, line 14, delete "2" and replace with --2A, 2B--.

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REVIEW

--92. (New) The method of claim 89, wherein the fibrin-containing substance is within blood vessels of a subject and wherein contacting is performed by administering the imaging agent

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contained in a suitable carrier to the subject under conditions permitting the imaging agent to enter the blood vessels of the subject.--

--93. (New) A method of claim 92, wherein the fibrin-containing substance is a thrombus.--

--94. (New) A method of claim 92, wherein the fibrin-containing substance is atherosclerotic plaque.--

--95. (New) An agent of claim 89, wherein the marker is a radioactive isotope, an element which is opaque to X-rays, or a paramagnetic ion.--

--96. (New) A method of claim 89, wherein the imaging is carried out using a gamma camera.--

In the Drawings

Please replace Figures 1A-53 on sheets 1/62 to 62/62 with replacement Figures 1-53E on sheets 1/86 to 86/86 attached hereto as Exhibit 1.

REMARKS

The subject application is a continuation of U.S. Serial No. 08/909,140, filed August 11, 1997, which is a divisional of U.S. Serial No. 08/409,750, filed March 24, 1997, which is a continuation of U.S. Serial 08/058,241, filed May 4, 1993. A Notice of Allowance and issue fee due was issued on September 14, 1999 in connection with U.S. Serial No. 08/909,140. On December 14, 1999, applicants paid the issue fee. Accordingly, U.S. Serial No. 08/909,140 is pending today and the subject application is co-pending therewith for the purposes of 35 U.S.C. §120.

By this Preliminary Amendment, applicants have amended the

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specification to provide an updated history of the parentage of prior related applications, to correct typographical errors and to provide deposit accession numbers at the appropriate locations in the subject application. Additionally, applicants have replaced the drawings as originally filed with formal drawings of Figures 1-53E. Applicants have also canceled claims 1-87 without prejudice and added new claims 88-96. Accordingly, claims 88-96 are presently under examination.

Support for the amendments to the specification may be found, inter alia, in the sentence or paragraph in which the amendment is made or in the drawings.

Support for new claim 88 may be found, inter alia, on page 45, line 32 to page 46, line 4 and on page 47, lines 15-23.

Support for new claims 89-95 may be found, inter alia, on page 47, line 26 to page 48, line 13.

Support for new claim 96 may be found, inter alia, on page 49, lines 7-9.

Applicants point out that the amendments made hereinabove to the specification and drawings merely conform the present specification and drawings to the allowed specification and drawings in the grandparent application, U.S. Serial No. 08/058,241, filed May 4, 1993. Applicants maintain that this Preliminary Amendment does not introduce new matter. Accordingly, applicants respectfully request entry of this Preliminary Amendment.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided.

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No fee, other than the enclosed \$690.00 filing fee, is deemed necessary in connection with this Preliminary Amendment. If any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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**Application
for
United States Letters Patent**

To all whom it may concern:

Be it known that we,

TIKVA VOGEL, AVIGDOR LEVANON, MOSHE WERBER, RACHEL GUY
AND AMOS PANET

have invented certain new and useful improvements in

FIBRIN BINDING DOMAIN POLYPEPTIDES AND USES AND METHODS
OF PRODUCING SAME

of which the following is a full, clear and exact description.

FIBRIN BINDING DOMAIN POLYPEPTIDES
AND USES AND METHODS OF PRODUCING SAME

5

This application is a continuation-in-part of U.S. Serial No. 345,952, filed April 28, 1989, which is a continuation-in-part of U.S. Serial No. 291,951, filed December 29, 1988, the contents of both of which are hereby incorporated by reference into this application.

10

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

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Atherosclerosis is the progressive narrowing of the lumen (inner passageway) of arterial blood vessels by layers of plaque (fatty and fibrous tissues). Atherosclerosis can occur in any artery. In coronary arteries it may result in heart attacks; in cerebral arteries it may result in strokes; and in peripheral arteries it may result in gangrene of the legs and feet.

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Atherosclerosis is the single largest medical problem currently facing the United States and other developed countries. Approximately 40 million people in the United States are at risk of developing atherosclerosis. However, only 6 million people in the United States show overt signs of the disease. The rest remain undiagnosed until the disease manifests

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itself symptomatically, in the worst case as heart attack or stroke. Heart attack and stroke, respectively, represent the first and third leading causes of death in the United States. Over 500,000 people die of heart attacks every year and a significant sub-group of these patients expire without warning.

The endothelium is located between the blood and arterial tissue and serves as a barrier against the accumulation of blood components in the vascular wall. Formation of atherosclerotic lesions (plaques) in the sub-endothelium is associated with major coronary artery disease and stroke. The causes and detection of such lesions have been intensely investigated.

Endothelial injury is believed to be an initial step in the formation of the atherosclerotic lesions and may be caused by, i.e., hemodynamic strain, hypercholesterolemia, hypertension and immune complex disease. Endothelial injury leads to thickening of the intima, cell proliferation, cholesterol accumulation, and formation of connective tissue fibers. IgG and complement factor C3 accumulation in injured endothelial cells and nonendothelialized intima has been observed. Mononuclear cells derived from blood are also part of the cell population in atherosclerotic lesions. The mechanism of plaque formation is not fully known. However, a probable mechanism is that the earliest lesions, fatty streaks, consisting of mixtures of T cells and monocyte-derived macrophages, form in the subendothelium followed by a secretion of various cytokines, which leads to a migration of smooth cells into the intima and their accumulation.

Although atherosclerosis is generally a diffuse disease, human coronary atherosclerosis lends itself to bypass procedures because the major site of plaque formation is usually proximally distributed. As a result, direct coronary artery bypass has become the most frequently selected form of myocardial revascularization. The aorta-coronary artery vein graft of the internal mammary artery graft have become technically standardized and have high long-term patency rates. These long-term results, however, can be compromised by progressive atherosclerotic lesion distal to the graft anastomosis. Other cases are inoperable because of distal disease. Previously, distal lesions have been ignored or, in selected cases, treated by endarterectomy although neither approach has proved entirely satisfactory.

Most existing procedures for the diagnosis and treatment of atherosclerosis are invasive, costly, and of limited effectiveness in a significant percentage of patient cases.

The concept of plaque enhancement by application of a stain has been reported [Spears, J. et al., J. Clin. Invest. 71: 395-399 (1983)]. These stains mark the plaque surfaces with a fluorescent compound. Plaque destruction by photoactivation of hematoporphyrin derivatives using an intraluminal laser-transmitting optical fiber has been suggested [Abela, G. et al., Am. J. Cardio. 50: 1199-1205 (1982)]. Moreover, tetracycline stains have also been suggested. [Murphy-Chutorian, D. et al., Am. J. Cardiol. 55: 1293-1297 (1985)].

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The above-identified stains were selected for their ability to bind to components of the atherosclerotic plaque. In principal, the stain absorbs laser light concentrating the light at the stained surface. Some staining of healthy tissue occurs causing stain associated damage to the surrounding tissue. Because laser light wavelength is limited to the absorption wavelength of the stain, chromophores offering optimum absorption of laser light must be used to provide best controlled ablation.

Imaging and detection of coronary thrombi, pulmonary emboli, deep venous thrombosis and atherosclerotic lesions are of great clinical importance especially in view of the new thrombolytic agents which have recently been developed. Several experimental approaches for non-invasive detection of thrombi by use of radiopharmaceutical agents have been reported but none has gained wide clinical recognition because of intrinsic drawbacks associated with each agent.

The basic characteristics of a radiopharmaceutical for early detection of intravascular atherosclerotic lesions and thrombi are the following: (i) high affinity for thrombus components; (ii) relatively fast pharmacokinetic blood clearance rate [in order to obtain a high ratio of thrombus (bound) to blood (unbound) radiolabeled tracer]; (iii) safety: non-toxic and non-immunogenic; and (iv) simplicity of preparation and use.

The various agents for imaging thrombi described in the literature and their drawbacks are as follows: (a) autologous platelets labeled with ^{111}In : the procedure is cumbersome, time consuming and the blood clearance

time is relatively long, viz. 2 days (2); (b) ^{131}I -fibrinogen: the assay is based on the (low) affinity of injected radiolabeled fibrinogen for the thrombus but it is not suitable for rapid imaging tests because of its long residence time in blood and furthermore it does not become incorporated into older thrombi nor is it incorporated in the presence of heparin (3, 36); (c) fragment E1 of human fibrin: although it seems superior to fibrinogen it is difficult to prepare in sufficient quantities for widespread clinical use (4); (d) mouse anti-fibrin monoclonal antibodies: although they are specific and have high affinities to thrombi they have a relatively long blood clearance time and are potentially immunogenic to human subjects (5, 33, 34); (e) mouse monoclonal antibodies specific for activated platelets (6, 7): disadvantage as (d); and (f) labeled fibronectin (1): although fibronectin (see below) has an affinity for a number of substances occurring in thrombi it has a relatively long blood clearance time and the buildup of radioactivity in the thrombus is slow. Thus there is a need in the art for a thrombus-specific radiopharmaceutical for rapid imaging of thrombi.

U.S. Patent 4,343,734 (Lian et al.) describes specific gamma-carboxyglutamic acid (GLA) antibodies which can be labeled with fluorescein for immunofluorescence staining of tissue to determine the presence therein of GLA. Specific GLA antibodies bind to GLA which is present in advanced atherosclerotic plaque, having calcium deposits. Lian et al. report that GLA is not found in uncalcified plaques and that GLA is found in cardiac valves and aortas, and in circulating proteins such as prothrombin, clotting factors VII, IX and X, Protein C and Protein S. However, the GLA binding

antibodies of Lian et al. do not selectively bind to atherosclerotic plaque.

5 Fibronectin is a glycoprotein composed of two identical subunits each of approximately 220,000 molecular weight. Two major forms of fibronectin are produced and secreted by human cells in culture and in vivo (8). The cell-associated fibronectin is relatively insoluble and participates in cell adhesion, wound healing, cell
10 differentiation and phagocytosis. The plasma fibronectin, produced primarily in the liver, is a soluble serum protein with biological properties similar to those of cell fibronectin.

15 Fibronectin is considered a multifunctional modular protein since limited proteolytic cleavage produces polypeptides with distinct activities. The different functional domains of the fibronectin molecule have been obtained and defined by partial proteolytic
20 digestion, and include heparin, DNA, fibrin, gelatin, and cell binding domains (8-13).

Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1989, discloses the complete cDNA
25 sequence of fibronectin. Baralle also discloses the expression of fusion proteins containing a portion of the collagen binding domain of fibronectin fused to the Escherichia coli protein β -galactosidase. Similar fusion proteins are disclosed by Owens and Baralle
30 (14). Obara et al. (1987) disclose the expression of a portion of the cell binding domain of human fibronectin fused to Escherichia coli β -galactosidase (15). Additionally, Obara et al. (1988) disclose the expression of portions of the cell binding domain fused to β -
35 galactosidase which have been mutagenized, i.e., site

specific deletions of portions of the cell binding domain were obtained as fused proteins (16). The carboxy terminal fibrin-binding domain of human fibronectin has been expressed in mouse L cells as a fusion protein with the signal sequence of human protein C inhibitor (17).

None of the above references discloses the expression of the N-terminal fibrin binding domain of fibronectin; furthermore all the recombinant proteins they disclose are expression of fusion proteins.

This invention provides polypeptides having an amino acid sequence substantially present in the N-terminal fibrin binding domain of fibronectin. These polypeptides have varying molecular weights (31 kD, 20 kD and 12 kD), as defined by comparison markers on SDS gels under reducing conditions, and have the following characteristics which make them promising pharmaceutical agents: (i) have an amino acid sequence present in a human protein and thus are contemplated to not be immunogenic; (ii) high affinity to fibrin and able to become covalently cross-linked to growing as well as to preformed thrombi (clots); (iii) bind to extracellular matrix, which property may be exploited to detect atherosclerotic plaques; (iv) have a relatively short blood clearance time; (v) incorporate into clots in the presence of heparin; and (vi) are produced by recombinant techniques and can therefore potentially be manufactured on a large scale.

The subject invention provides an inexpensive, accurate method for imaging fibrin-containing substances, i.e., a thrombus and atherosclerotic plaque, both in vitro and in vivo. In addition, the subject invention

provides plasmids for expressing polypeptides having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and capable of binding to fibrin which are labeled and used for imaging the fibrin-containing substances, and methods of producing such polypeptides.

These polypeptides may also be used as anti-infective agents. The involvement of fibronectin in adhesion to, and invasion of, wounds by a wide range of gram positive bacteria is well established (18, 19). The polypeptides of the fibrin binding domain of fibronectin according to this invention may be used as anti-infective agents to prevent sepsis in wounds.

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Summary of the Invention

This invention provides an imaging agent which comprises a polypeptide labeled with an imageable marker, such polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

Further provided is a method for imaging a fibrin-containing substance, i.e. a thrombus or atherosclerotic plaque, which comprises contacting the fibrin-containing substance to be imaged with the imaging agent disclosed above under conditions such that the agent binds to the fibrin-containing substance and imaging the bound agent and thereby imaging the fibrin-containing substance.

Also provided is a plasmid for expression of a polypeptide which having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

The invention also provides a purified polypeptide substantially free of other substances of human origin which has an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

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Brief Description of the Figures

In the figures, the numbers in brackets adjacent certain of the restriction enzyme sites shown correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA as shown in Figure 1 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

The following figures describe the construction of plasmids expressing polypeptides having an amino acid sequence substantially present in the amino-terminal fibrin binding domain (FBD) of fibronectin. The FBD commences at amino acid number 1 of mature fibronectin, which is glutamine and corresponds to the fourth amino acid (G) shown in Figure 1A, i.e., the N-terminus of the FBD sequence is G-A-G-G (glutamine-alanine-glutamine-glutamine); the corresponding first nucleotide in the cDNA sequence of Figure 1A is therefore number 14, indicated by an arrow. All the recombinant FBD polypeptides described in these figures and throughout the specification are numbered from this first glutamine as amino acid number 1 and all the corresponding cDNA sequences are numbered as shown in Figure 1.

Some of the figures describe the construction of plasmids expressing an FBD polypeptide joined at its C-terminus to part of the cell binding domain (CBD) of fibronectin. The cDNA sequence corresponding to the CBD which applicants have cloned and expressed is missing the 270 bp extra domain (ED) segment which extends from nucleotides 4812 to 5081, inclusive, on the Baralle map (see Figure 1). Thus, the cDNA

sequence which is said to extend from nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because it is missing the 270 nucleotides of the ED segment, namely from nucleotides 4812 to 5081 inclusive; this region is also known in the art as the ED-A region; concomitantly amino acid 1690 is changed from alanine to threonine. Similarly, the polypeptide expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on the Baralle map but would be missing the 90 amino acids encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would contain only 660 amino acids. This is true for all CBD polypeptides described in this application which span the ED region. (The region known in the art as the ED-B region is missing both in Baralle's sequence and in applicants' DNA.)

The definition of the polypeptides expressed as 31 kD, 20 kD, 12 kD and 33 kD is an operational definition, based on their mobility on SDS polyacrylamide gels under reducing conditions compared to that of markers of known molecular weight.

Figure 1. This figure shows the nucleotide sequence of human fibronectin cDNA.

Figure 2. Seven pairs of chemically synthesized oligomers were prepared. The synthetic oligomers code for the first 153 N-terminal amino acids of human fibronectin (FN). This figure shows the sequence of these 7 pairs of synthetic oligomers.

Figure 3. The DNA fragment coding for amino acids 1 to 153 of the N-terminal domain of human FN was

assembled from the 7 pairs of chemically synthesized oligomers shown in Figure 3 as follows:

Oligomers 3/4, 5/6, 7/8 and 9/10, each pair in a separate tube, were annealed and then phosphorylated at the 5' end using T4 polynucleotide kinase enzyme.

In the second step, pairs 3/4 and 5/6 were ligated to each other using T4 DNA ligase. Similarly, reaction pairs 7/8 and 9/10 were ligated to each other. After each step of ligation an aliquot of the ligation mixture was analyzed on gel to determine the size of the newly formed fragments and the efficiency of ligation.

In the third step, the two above mentioned ligation mixtures were mixed together and pair 6, oligomers 11/12 which had been annealed and phosphorylated previously in a separate tube were added to the mixture. A 326 base pair DNA fragment obtained from the above ligation mixture was isolated from an agarose gel and purified.

The purified synthetic 326 fragment was added to two additional pairs of synthetic linkers: Pair 1, oligomers 1/2 and Pair 7 oligomers 13/14. In Pair 1 only oligomer 2 was phosphorylated at the 5' end and in Pair 7 only oligomer 13 was phosphorylated at the 5' end.

After ligation with T4 DNA ligase the mixture without any further isolation was added to pBR322 vector DNA digested with EcoRI and BamHI endonucleases.

The plasmid obtained, designated pFN 932-18 contained the entire synthetic EcoRI (5' end) - BamHI (3' end) restriction fragment coding for the N-terminal 153 amino acids of human FN, in a pBR322 vector.

5 Figure 4. Expression of the N-terminal 153 amino acid sequence of FN.

10 Plasmid pFN 932-18 was digested with NdeI and BamHI endonucleases. The NdeI-BamHI DNA fragment coding for FN (first 153 amino acids + additional N-terminal methionine) was isolated and ligated into the large fragment obtained by digestion of plasmid pTV301 with NdeI and BglII endonucleases. (Plasmid pTV301 (Figure 15 33) expresses human growth hormone, hGH, under the control of λ P_L promoter and the cII RBS).

The plasmid obtained was designated pFN949-2.

20 Figure 5. Insertion of termination codon TAA at the 3' end of the N-terminal domain of FN (at amino acid 262)

25 A synthetic oligonucleotide containing a TAA termination codon and a BglII site having the following sequence:

CTGTTTAAGCA
GACAAATTCGTCTAG

30 was ligated to the 3' end (PvuII site) of an EcoRI-PvuII FN fragment isolated from cDNA clone plasmid p931-5 (see Figure 5) digested with EcoRI and PvuII. The ligation was carried out in the presence of DNA 35 vector plasmid pBR322 digested with EcoRI and BamHI

(large fragment). The plasmid obtained was designated pFN935-12.

5 Figure 6. Subcloning of the carboxy-terminal
 region of FBD in λ P_i expression vector

10 Plasmid pFN 935-12 was digested with EcoRI and HincII.
 The EcoRI-HincII fragment coding for FN was isolated
 and ligated to DNA, the large fragment obtained by
 digestion of plasmid pTV194-80 with EcoRI and SmaI.
 (Plasmid pTV194-80 expresses human ApoE under the
 control of the λ P_i promoter and β -lactamase promoter
 and RBS). The plasmid obtained was designated pFN 946-
15 12. This plasmid is deleted of the PBLA sequences and
 therefore does not express the carboxy domain of FBD.

 The construction of pTV194-80 from plasmid p579 (Figure
 34) is shown in Figures 30 and 31.

20 Figure 7. Construction of the DNA fragment coding
 for the FBD from nucleotide No. 14 to
 nucleotide No. 599

25 Three pairs of chemically synthesized oligomers with
 the following DNA sequences:

Pair 1

 #15 5'-TGAGAAGTGTTTGTGATCATGCTGCTGGGACTTCCTATGTGG-3'

 #16 3'- CTTCAAAAAGTAGTACGACGACCCTGAAGGATACACCAGCCT-5'

30 Pair 2

 #17 5'-TCGGAGAAACGTGGGAGAAGCCCTACCAAGGCTGGATGATGGTAG-3'

 #18 3- CTTTGACCCCTCTTCGGGATGGTTCCGACCTACTACCATCTAACA-5'

Pair 3

#19 5'-ATTGTA CTTGCCTGGGAGAAGGCAGCGGACGCATCACTTGCACTT-3'

#20 3'- TGAACGGACCCTCTTCCGTCGCCTGCGTAGTGAACGTGAAGATC-5'

were used to carry out this construction.

Oligomers 15/16 and 17/18 were annealed and phosphorylated at the 5' end each in a separate tube and then mixed together for ligation using T4 DNA ligase. After 3 hours of ligation, oligomers 19/20 (previously annealed and kinased at their 5' ends) were added for an additional 3 hours ligation at room temperature.

The synthetic DNA fragment obtained was used for further ligation with an EcoRI-DdeI FN coding sequence obtained from plasmid pFN 932-18 digested with EcoRI and DdeI. The ligation was carried out in the presence of plasmid pUC19 digested with EcoRI and XbaI (large fragment).

The plasmid obtained was designated pFN948-4.

Figure 8. Construction of the entire FBD region

Plasmid pFN948-4 was digested with EcoRI and XbaI. The EcoRI-XbaI fragment coding for the N-terminal region of FBD was isolated and ligated to the carboxy terminal region of FBD by digestion of plasmid pFN946-12 with EcoRI and XbaI (using the large fragment). The plasmid obtained was designated pFN 957.

Figure 9. Expression of the entire FBD polypeptide
under the control of the λ P_L promoter
and λ cII RBS

Plasmid pFN 957 was digested with NdeI and HindIII. The NdeI-HindIII fragment coding for the FBD was isolated and ligated into the isolated vector fragment of plasmid pTV301 digested with NdeI and HindIII in the presence of isolated purified HindIII-HindIII T₁T₂ coding DNA fragment. The plasmid obtained was designated pFN962-3.

Figure 10. Expression of the entire FBD polypeptide
under λ P_L promoter and PBLA ribosomal
binding site

Plasmid pBLA11 (ATCC Accession No. 39788) was digested with EcoRI and AluI. The EcoRI-AluI fragment coding for the β -lactamase promoter and β -lactamase RBS was isolated and ligated into plasmid pFN962-3 (Figure 9) digested with NdeI, then treated with Klenow enzyme in the presence of all four dNTPs to fill in the NdeI site and digested with EcoRI (using the large fragment). The plasmid obtained was designated pFN 975-25.

Figure 11. Refolding/reoxidation and purification of
r31 kD as followed by SDS-PAGE under
reducing and non-reducing conditions

The gel (12% acrylamide) under reducing conditions (with β -mercaptoethanol (ME)) monitors the process of purification, whereas the non-reducing conditions (without ME) are indicative of the refolding/reoxidation, leading to faster moving and less diffuse bands. Note (in the absence of ME) that the band of 'After Phenyl-S' is much

sharper than that of 'Refolded', indicating that reoxidation continues even during the purification.

5 Refolded (GSH/GSSG):r31kD which has been refolded/re-oxidized - after having been extracted from the crude pellet in the presence of GSH/GSSG 3 mM/0.3 mM at pH 8.0; Phenyl-S; Phenyl-Sepharose; Q-S; Q-Sepharose; p31 kD; plasma-derived 31 kD (obtained by tryptic digestion); molecular weight markers: Low Molecular Weight protein calibration kit (Pharmacia Fine Chemicals), containing markers whose molecular weights are 94 kD, 67 kD, 43 kD, 30 kD, 20.1 kD and 14.4 kD.

10
15 Figure 12. Purification of GSH/GSSG-refolded r31 kD by Phenyl-Sepharose chromatography

20 A suspension of refolded/reoxidized and "scrambled" 31 kD, as well as insoluble contaminants, which has been extracted from 10 grams of pellet, was subjected to centrifugation at 13,000 rpm (see Section 3.1 in Example 5). The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A at pH 8.5, containing also 0.2 M AS. The column was washed
25 with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl. The purified r31kD appeared in the Buffer A fraction and at this stage it was more than 85% pure. Absorbance was measured at 280 nm.

30
35 Figure 13. Concentration and purification of r31 kD by Heparin-Sepharose chromatography

Approximately 1/2 of the Buffer A peak from the phenyl-Sepharose step (see Figure 12) was concentrated and

purified on a 10 ml Heparin-Sepharose column, from which it was eluted by a solution of 0.5 M NaCl in Buffer A. At this stage the r31 kd is more than 90% pure. Absorbance was measured at 280 nm.

5

Figure 14. Purification of r31 kd by Q-Sepharose chromatography

10

Concentrated r31 kd (Figure 13), which had been dialyzed against Buffer A, pH 8.5, was loaded on a 40 ml column of Q-Sepharose, which had previously been equilibrated with the same buffer. The purified r31 kd, which eluted in the flow-through and wash fractions, was concentrated by lyophilization. The column was washed free from the contaminant proteins by a step of 1 M NaCl. The purified r31 kd is at this stage more than 95% pure. Absorbance was measured at 280 nm.

15

20

Figure 15. Analytical FPLC-gel permeation of r31 kd on a Superose 12 column

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A mixture of plasma derived and recombinant 31kd (100 μ l at 0.8 mg/ml) in running buffer (20 mM Tris.HCl - 150 mM NaCl. pH 7.8) was applied onto the Superose 12 column (HR 10/30), pre-equilibrated in running buffer, and eluted from it in the same buffer. Flow rate - 0.4 ml/minute; chart speed - 0.25 cm/minute; absorption units full scale - 0.1; detection wavelength - 280 nm; run time - 60 minutes. The retention times of both the plasma derived and recombinant 31kd, when run separately, was identical to that obtained for the mixture, and correspond to an apparent molecular weight of 37 kD.

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Figure 16. Comparison of the pharmacokinetics of fibronectin (FN) and r31 kD FBD in rats

¹²⁵I-FN (0.1 mg/kg; 5 x 10⁶ cpm) or ¹²⁵I-r31 FBD (0.1 mg/kg; 5 x 10⁶ cpm) were injected intravenously and at the time indicated blood samples were withdrawn. Insoluble radioactivity in the blood samples was determined by trichloroacetic acid precipitation; at zero time, the 100% value represents 40,000 cpm/ml for FN and 46,000 cpm/ml for FBD.

Figure 17. Binding of ¹²⁵I-r31 kD to fibrin: effect of thrombin and Ca⁺⁺ ions

Reaction I was carried out as described in Example 7 using 20 μ l citrated whole blood, 0.15 μ M ¹²⁵I-r-FBD (r31 kD) (5.6 x 10⁵ cpm/ μ g).

Reaction II was initiated with the formation of unlabeled Fibrin clot using 20 μ l citrated whole blood as described in Example 7. After the first incubation, 0.15 μ M ¹²⁵I-r-FBD was added to the existing reaction tube ("Serum") or to the Fibrin pellet following centrifugation and resuspension in PBS ("PBS"). When CaCl₂ and Hirudin were added the concentrations were 5 mM and 3 U/ml, respectively. Reaction II was continued thereafter as described in Example 7.

Figure 18. Release of ¹²⁵I-FBD (p31 kD) from Fibrin clot by plasmin

Reaction I was carried out using several tubes containing 100 μ l citrated whole blood and 0.3 μ M ¹²⁵I-P-FBD (5.0 x 10⁴ cpm/ μ g). At the end of the incubation, the pellet

was collected by centrifugation and then resuspended in PBS solution containing 1 μ /ml plasmin (from porcine blood, Sigma) and was further incubated at 37°C for the indicated time intervals. The reaction was terminated by cooling and immediate centrifugation. The radioactivity in the supernatant and the pellet was measured by a gamma counter.

The pellet and the supernatant were resuspended in gel electrophoresis sample buffer (final concentration of 3% glycerol, 2% BME, 1% SDS, 0.2% Bromophenol Blue), boiled for 15 minutes, and electrophoresed in 10% PAGE-SDS. The gel was then autoradiographed on x-ray film. No radioactivity was detected from the pellet. Radioactivity from the supernatant was detected in a position corresponding to control 125 I-FBD incubated with plasmin (results not shown).

Figure 19.

The binding of 0.15 μ M 125 I-r-FBD (125 I-r31 kD; 5.6×10^5 cpm/ μ g) to 20 μ l citrated whole blood was performed using reaction I conditions (see Example 7). The reaction was performed either without competitors (C) or in the presence of various concentrations of unlabeled "folded proteins", "reduced" FBD and related molecules.

rI	=	"Fully reduced" r31 kD FBD
rII	=	"Reduced carboxyamidated" r31 kD FBD (i.e. reduced/blocked 31 kD FBD)
p	=	31 kD FBD from Trypsin cleavage of plasma derived Fibronectin.

Figure 20. Binding of ^{125}I -r31 kD to fibrin (Reaction II). Effect of transglutaminase inhibitors and related molecules

5 Unlabeled fibrin clot was formed using 20 μl citrated whole blood and using the conditions described for reaction II in Example 7.

10 At the end of the first incubation period, and prior to the addition of r- ^{125}I -FBD ($0.15 \mu\text{M}$; $2.9 \times 10^4 \text{ cpm}/\mu\text{g}$), the indicated concentrations of spermidin, putrescine and FBD-"R" ("Reduced-carboxamidated" plasma derived FBD) were added to the specified reactions.

15 Figure 21. Binding of ^{125}I -r31 kD to preformed fibrin clot (Reaction II): effect of fibrin clot age

20 The unlabeled fibrin clot was formed using 20 μl citrated whole blood and using the conditions described for reaction II in Example 7.

25 At the end of the first incubation period, half of the samples were centrifuged and the fibrin pellet was resuspended in PBS solution containing 5 mM CaCl_2 (Reaction "C") or PBS containing 5 mM CaCl_2 and 300 $\mu\text{g}/\text{ml}$ FN (Reaction "D"). No additions or changes were performed to the samples designated "A". 300 $\mu\text{g}/\text{ml}$ FN was added to the sample designated "B". Subsequently, the reaction
30 mixtures were allowed to incubate at 37°C for 1 hour, 4 hours, or 24 hours (as indicated in the figure). Then ^{125}I -r31 kD ($0.15 \mu\text{M}$ final concentration, $5.4 \times 10^5 \text{ cpm}/\mu\text{g}$) was added and the incubation was continued for an additional 30 minutes. The reaction was terminated as
35 described in Example 7.

Figure 22. Binding of ^{125}I -r31 kD to "Naive" Thrombi (reactions I and II): effect of thrombi age on the binding

5 Aliquots of 20 μl non-citrated fresh whole human blood were incubated in non-siliconized tubes at 37°C with either 0.15 μM ^{125}I -r31 kD or alone (reaction I and II, respectively). At the indicated time intervals, reactions were either terminated (reaction I) or 0.15 μM ^{125}I -r31 kD (2.9×10^4 cpm/ μg) was added, and incubation terminated after an additional 2 hours (reaction II).

Figure 23. Binding of ^{125}I -r31 kD to Fibrin (Reaction I): Effect of Exogenous Transglutaminase and "Reduced" FBD

15 20 μl aliquots of noncitrated whole human blood were incubated with 0.15 μM ^{125}I -r31 kD (2.9×10^4 cpm/ μg) alone ("control") or together with pig liver Transglutaminase ("control + T.G.", 0.2 units/ml; Sigma).

20 Some of the tubes contained "Reduced" (carboxamidated) p31 kD as indicated in the figure.

25 The addition of exogenous Transglutaminase to the binding reaction increased the binding values by more than a factor of two. When "reduced-carboxamidated" r31 kD was added to the reaction we observed a similar extent of inhibitory effect as with the exogenous factor XIIIa (inhibition of 53% and 71% by 0.3 μM and 3.0 μM , respectively), indicating an identical inhibitory effect of the reduced FBD on both types of Transglutaminase.

Figure 24. Binding of FBD to ECM

The biological activity of the FBD was studied in a model of vascular injury using the Extra Cellular Matrix, "ECM", of cultured endothelial cells (20).

33 mm ECM plates following 3x washing in PBS were incubated at 37°C in a CO₂ incubator with 0.5 ml DMEM-10% FCS containing 2.5 mM CaCl₂, 1 µ/ml Thrombin, and the indicated concentrations of ¹²⁵I-P-FBD (about 4.4 x 10⁵ cpm/µg). Parallel plates were incubated in the absence of thrombin as indicated in the figure. Following 45 minutes of incubation, the plates were washed 3 times with 1 ml PBS, extracted with 0.5 ml of 0.1% SDS-PBS solution, and radioactivity was measured by a gamma counter. The values described in the figure represent an average of two plates.

Figure 25. Binding of FN and FBD to S. aureus

The binding reaction was carried out in a solution using 5 x 10⁸ PFU/ml of S. aureus SA113 (ATCC Accession No. 35556) and ¹²⁵I-FN (4 x 10⁴ cpm/µg) or ¹²⁵I-FBD (1.3 x 10⁵ cpm/µg; r31 kD FBD, "reoxidized-refolded") at concentrations indicated in the figure and as described in methods. The concentration of the labeled molecules described is calculated using molecular weights of 220,000 and 31,000 daltons for FN and r31 kD, respectively.

Figure 26. Binding of FBD to S. aureus: competition with folded and reduced forms

5 Binding in solution of 1.25 μ g 125 I-p31 kD (2.3×10^5 cpm/ μ g) to 5×10^8 PFU/ml of S. aureus SA113 was carried out in the presence of the indicated concentrations of the following unlabeled proteins: P-FBD (p31 kD), r-FBD (r31 kD FBD "reoxidized-refolded"), r-FBD-R (r31kD FBD "Reduced Carboxyamidated), and r-CBD (r-33 kD cell binding domain of FN). The binding reaction was carried out as indicated in the methods section.

Figure 27. Binding of S. aureus to immobilized FN

15 Binding of 7.5×10^8 PFU/ml of 3H-leucine-S. aureus (5.8 cpm/ 10^5 PFU) to FN immobilized onto plastic vials was carried out as described in methods and in the presence of human plasma FN, r-FBD (r31 kD FBD "reoxidized-refolded"), P-FBD (p31 kD), or BSA (Bovine Serum Albumin, Sigma). Binding of "control" reaction in the absence of competitors (9.3% of input bacteria) was normalized to 100%.

Figure 28. Binding of S. aureus to Catheters

25 Binding of 3.0×10^6 PFU/ml of 125 I-S. aureus (1 CPM/3 PFU) to "Uno" bronchial plastic catheters (3 cm for each reaction, in duplicate) coated with FN was carried out as described in methods. When competition reaction was performed, the bacteria and the added protein were pre-incubated at room temperature for 30 minutes and then added to the catheters for further incubation as described in the methods section.

The polypeptides used in the competition reactions were: P-31 (p31 kD), r-20 (recombinant derived 20 kD FBD) and r-31 (reoxidated and refolded r31kD). Some of the reactions (see figure) were measured in the presence of 5 μ m Heparin (from porcine intestinal mucosa, molecular weight of 10,000; Sigma).

The binding of "control" reaction in the absence of competitors (8.8% of input bacteria) was normalized to 100%.

Figure 29. Adhesion of p31 kD in Rabbit Aorta Lesion Model

The figure shows the distribution of radioactivity in serially sectioned aorta segments from balloon catheterized rabbits. The measurements were taken 72 hours after injection of 125 I-labeled Fibronectin (FN) or plasma derived 31kD FBD (31kD).

Figure 30. Construction of pTV-170

The NdeI-NdeI ApoE fragment was isolated from plasmid pApoE-EX2 (ATCC Accession No. 39787) and inserted into the unique NdeI site of the expression vector p579 (Figure 34) which had been digested with NdeI. The resulting plasmid pTV-170 expresses an analog of natural human ApoE protein having a methionine residue added at the N-terminus.

Figure 31. Construction of pTV-194-80

The β -lactamase promoter and ribosomal binding site fragment was isolated from plasmid pBLA11 (ATCC Accession No. 39788) after digestion with EcoRI and AluI. This fragment was ligated to the large fragment of pTV-170

(Figure 30) plasmid which had been digested with NdeI, filled in with DNA polymerase I (Klenow) and then digested with EcoRI.

5 Figure 32. Recombinant polypeptides of fibronectin domains compared to full-length fibronectin

10 This figure shows the alignment of cDNA clones encoding various recombinant polypeptides relative to one another and to the full-length sequence of fibronectin cDNA and to a schematic representation of the various domains present within the human fibronectin molecule.

15 Figure 33. Construction of plasmid pTV301 from plasmids pTV104(2) and p579

20 Plasmid pTV301 directs the expression of a human growth hormone analog under the control of the λ P_L promoter and the cII ribosomal binding site. The plasmid also contains a T₁T₂ transcription terminator downstream of the cDNA encoding human growth hormone.

25 Figure 34. Construction of plasmid p579

30 The rRNA operon T₁T₂ transcription termination fragment was isolated from plasmid pPS1 (deposited with the ATCC under Accession No. 39807) which had been digested with HindIII. The T₁T₂ fragment was inserted into the unique HindIII site of pRO211 (Figure 35) which had been digested with HindIII. The resulting expression vector, p579, contains the λ P_L promoter and the C₁₁ ribosomal binding site, followed by the T₁T₂ transcription termination sequences.

Figure 35. Construction of plasmids pRO211 and pRO12

The plasmid pJH200 (deposited with the ATCC under
Accession No. 39783) was partially digested with NdeI,
treated with DNA polymerase I (Klenow) to fill in the
ends and the resulting ends were religated to form the
expression vector pRO211. The expression vector pRO211
was digested with NdeI and HindIII, the large fragment
isolated and ligated to an NdeI-HindIII bovine growth
hormone (bGH) fragment isolated from pAL500 (deposited
with the ATCC under Accession No. 39782) to give pRO12.
(The NdeI-HindIII fragment was produced from pAL500 by
digesting it with EcoRI and ligating to the ends of the
digestion product synthetic linkers with the sequence:

TATGGATC
ACCTAGTTAA

The ligation mixture was digested with NdeI and HindIII
and the resulting NdeI-HindIII bGH fragment isolated).

Figure 36. Construction of plasmid pFN 196-2 which
expresses the r12 kD FBD polypeptide

The large BspMI-HindIII fragment obtained by digestion of
plasmid pFN 975-25 (Figure 10) with BspMI and HindIII was
ligated by T4 DNA ligase to the synthetic pair of linkers
A (see Figure 41). Plasmid pFN 196-2 was produced,
transformed into Escherichia coli strain A1645 and
retransformed into Escherichia coli strain A4255.
Plasmid pFN 196-2 contains the 5'-terminal sequence of
fibronectin cDNA from nucleotide 14 to nucleotide 340,
i.e., it encodes the first 109 amino acids of the FBD of
fibronectin terminating with an arginine residue; it is

not yet known if an additional N-terminal methionine is present in the final polypeptide. Plasmid pFN 196-2 gives good expression of an r12 kD FBD polypeptide under the control of the λ promoter and β -lactamase ribosomal binding site, and has been deposited in the ATCC under Accession No. _____.

Figure 37. Construction of plasmid pFN 197-10, which expresses a modified 12 kD FBD polypeptide (12 kD')

Plasmid pFN 975-25 was treated as described in Figure 36 except that a different pair of linkers, B (see Figure 41) was used. The ligation produced plasmid pFN 197-10 which encodes the N-terminal sequence of the FBD of FN; however, a modification after nucleotide 340 to produce an NdeI site (CATATG) before the stop codon results in the encoding of a polypeptide containing 111 amino acids where the first 109 amino acids correspond to those of the r12 kD polypeptide followed by two additional amino acid residues, viz. histidine and methionine; it is not yet known if an additional N-terminal methionine residue is present in the final polypeptide. Plasmid pFN 975-25 was transformed into Escherichia coli strain A1645 and hence into Escherichia coli strain A4255, and gave good expression of a modified r12 kD (12 kD') FBD polypeptide under the control of the λ promoter and the β -lactamase ribosomal binding site.

Figure 38. Construction of plasmid pFN 202-5 which expresses r12 kD' FBD fused to the r33 kD cell binding domain (CBD) of fibronectin

The large fragment produced after NdeI and HindIII digestion of plasmid pFN 197-10 (Figure 37) was ligated

by T4 DNA ligase to the NdeI-HindIII CBD (cell binding domain) fragment of plasmid pFN 137-2. Plasmid pFN 137-2, deposited in the ATCC under Accession No. 67910 has been described in the parent patent application, U.S. Serial No. 345,952; the r33 kD CBD sequence contains amino acids numbered 1329-1722 of fibronectin (see Figure 1) excluding the 90 amino acids numbered 1600-1689 encoded by the ED-A region (see preface to Brief Description of the Figures).

The resulting plasmid, pFN 202-5, was transformed into Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 202-5 contains the cDNA sequence of the 111 amino acids encoded by plasmid pFN 197-10 followed by the cDNA sequence for r33 kD CBD commencing with the codon for serine (the first amino acid of the r33 kD CBD). This plasmid gave good expression of an approximately 45 kD polypeptide comprising the r12 kD fibrin binding domain and the 33 kD cell binding domain of fibronectin; this fused polypeptide was expressed under the control of the λ promoter and the β -lactamase ribosomal binding site.

Figure 39. Construction of plasmid pFN 195-4 which expresses the r31 kD FBD fused to the sequence DGRGDS

The large fragment obtained by digestion of plasmid pFN 975-25 (Figure 10) with PvuII and HindIII was isolated and ligated with T4 DNA ligase to a pair of synthetic linkers, C (see Figure 41). The resulting plasmid, designated pFN 195-4 was transformed into Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 195-4 contains the full-length FBD cDNA sequence from nucleotide 14 to nucleotide 793

(encoding 260 amino acids) followed by a sequence encoding asp-gly-arg-gly-asp-ser, i.e., the polypeptide encoded has a total of 260 amino acids followed by the sequence DGRGDS; it is not known if an additional N-terminal methionine residue is present in the final polypeptide. Plasmid pFN 195-4 is a good expressor of the r31 kD fibrin binding domain fused to the sequence asp-gly-arg-gly-asp-ser (DGRGDS), under the control of the λ promoter and the β -lactamase ribosomal binding site.

Figure 40. Construction of plasmid pFN 194-2 which expresses a fused 31 kD FBD-33 kD CBD polypeptide

The large PvuII-HindIII fragment produced by digestion of plasmid pFN 975-25 (Figure 10) with PvuII and HindIII was isolated and ligated with T4 DNA ligase to a pair of linkers, D (Figure 41) and then ligated to the cell binding domain (CBD) fragment obtained by digestion of plasmid pFN 137-2 (ATCC Accession No. 67910) with NdeI and HindIII; see Figure 38 for definition of the CBD domain. The resulting plasmid, designated pFN 194-2, was transformed to Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 194-2 is a low expressor of a fused r31 kD FBD-r33 kD CBD polypeptide of approximate molecular weight 64 kD, under the control of the λ P_L promoter and the β -lactamase ribosomal binding site. The polypeptide encoded by plasmid pFN 194-2 contains DNA encoding the first 265 amino acids of fibronectin fused to a methionine codon, followed by the cDNA sequence for the CBD of fibronectin, commencing at the codon for amino acid serine at position 1 of the CBD.

Figure 41. Oligonucleotide linkers used in construction of plasmids

5 Four pairs of chemically synthesized oligomers (A, B, C and D) were prepared and were used to construct plasmids as described in Figures 36, 37, 39 and 40, respectively).

Figure 42. Uptake of labeled r31 kD FBD by stainless steel coil-induced venous thrombi

10 The bars and vertical brackets represent the mean \pm SEM (N=10) of the specific radioactivity associated with isolated thrombi, vein segments carrying the thrombus ("thrombi in-situ"), or peripheral blood samples 24h after administration of 125 I-31 kD FBD. For details, see Example 12, Section A.

Figure 43. Comparison of labeled r12 kD, r20 kD and r31 kD FBD polypeptides in the rat venous thrombus model

20 The bars and vertical brackets represent the mean \pm SEM (N=5) of the specific radioactivity associated with isolated thrombi (T) or blood (B) 24 hours after administration of the 125 I-labeled recombinant polypeptides, as indicated. For details, see Example 12, Section B.

Figure 44. Metabolic stability of 125 I-labeled r31 kD FBD in rats

25 Rats were injected intravenously with 125 I-r31 kD FBD (5×10^6 cpm/rat) in a similar experiment to that described in Figure 16. At the time intervals indicated blood samples were removed, placed in Na citrate containing

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tubes (final N-citrate concentration = 0.38%) and blood aliquots resulting were directed as follows: either (a) treated with 20% TCA and the TCA insoluble counts (after TCA precipitation) were measured; or (b) incubated with preformed clot (using 20 μ l whole blood from control rat); binding of the 125 I-31 kD FBD to the preformed clot was measured under the conditions of the two-step reaction II (Example 11). The radioactivity was measured by a gamma counter and the activity of each sample was calculated as a percentage of total cpm present in the reaction mixture. (Normally TCA precipitation includes placing sample aliquots on filters which are counted for total cpm, washing the filter 3 times with 20% TCA followed by twice with 20% ethanol to extract TCA, and recounting filters for TCA insoluble counts whereby the percentage of TCA-insoluble counts can be calculated.)

Figure 45. Specificity of binding to fibrin: effect of transglutaminase on the binding of r31 kD to a fibrin clot

The binding of 14 C-putrescine-r31 kD, 125 I-r31 kD FBD and 125 I-recombinant growth hormone in the presence or absence of transglutaminase was tested as described in Example 11, B. The radioactivity of the washed fibrin pellet was measured using a β -counter for the 14 C labeling and a gamma counter for the 125 I labeling and the ratio of counts in the presence and absence of transglutaminase was calculated for each protein.

Figure 46. Characterization of r31 kD FBD - fibrin complex by SDS polyacrylamide gel electrophoresis

¹²⁵I-r31 kD FBD was incubated with preformed fibrin clot derived from either 20 μ l whole human blood (A) or 250 μ l of 0.8 μ M solution of human fibrinogen (B). In one of the experiments (B) dental coils were added to the tubes together with the fibrinogen.

Binding of ¹²⁵I-r31 kD fibrin clot was measured using the two step Reaction II as described in Example 11 using 0.15 μ M ¹²⁵I-r31 FBD in the presence of 5 μ M CaCl₂. The reaction was terminated by washing three times with PBS and the pellet, after the various treatments described below, was centrifuged. 15 μ l aliquots of the supernatant were electrophoresed in SDS-polyacrylamide gels (15% gel). An autoradiogram was developed which is shown in the figure. The tracks of the gel are as follows:

- A.
1. plasmatic FN (220 kD)
 2. r31 kD FBD
 3. r31 kD FBD incubated for 1 hour with 2 units/ml plasmin (Sigma)

4-6. Binding experiments with rFBD incubated with preformed fibrin clot derived from citrated whole blood.

4. ¹²⁵I-r31 kD - fibrin pellet was boiled in sample buffer for 10 minutes (Sample buffer = 0.7 M β -mercaptoethanol/3% SDS/60 mM tris HCl, pH 6.8).

5. Binding conditions as described above with the addition of 0.02 units/ml guinea-pig liver transglutaminase (Sigma); pellet treated as in 4.

5

6. Binding conditions as described in (5); the pellet was incubated with 2 units/ml plasmin for 1 hour.

10

- B. 1-3. Experiments with r31 kD FBD incubated with preformed fibrin clot derived from pure human fibrinogen in the presence of transglutaminase.

15

1. Boiling of ^{125}I -r31 kD - FBD - fibrin pellets in sample buffer.

2. Boiling of ^{125}I -r31 kD - FBD - fibrin pellets in phosphate - saline buffer.

20

3. Boiling of ^{125}I -r31 kD - FBD - fibrin pellets in sample buffer plus 4 M urea.

25

- 4-5. Experiments as B. 1-3 above, but clots were formed in the presence of dental coils. Coils containing the clots were removed to different tubes and then the binding of ^{125}I -r31 kD to the clot was measured. The binding reaction was terminated by removal of the coils and analysis of the ^{125}I -r31 kD - fibrin complex attached to the coils.

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4. Boiling of ^{125}I -r31 kD - FBD - fibrin pellet in sample buffer.

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5. Boiling of ^{125}I -r31 kD - FBD - fibrin pellet in sample buffer plus 4 M urea.

Figure 47. Binding of ^{125}I -r31 kD FBD to preformed fibrin clots: effect of fibronectin (FN) and heparin

Experiments A and B were performed essentially as described for the two step Reaction II in Example 11.

A. Fibrin clots were formed during a 45 minute incubation period using 20 μl citrated whole blood, 5 mM CaCl_2 , 1 unit/ml thrombin in a final volume of 250 μl . The fibrin pellets, after centrifugation, were placed in 200 μl PBS - 5 mM CaCl_2 solution with or without purified plasma-derived FN (1 μM) and ^{125}I -r31 kD FBD (0.15 μM , specific activity 4×10^5 cpm/ μg). The binding of ^{125}I -r31 kD was determined after an additional incubation period of 30 minutes by measurement of radioactivity by a gamma counter.

B. Fibrin clots were formed as indicated in A. At the end of the first incubation period, ^{125}I -r31 kD FBD (0.15 μM , specific activity 3×10^4 cpm/ μg) was added together with various concentrations of heparin of average molecular weight 10,000 daltons (Laboratoire Choay, Paris, France; stock solution 5000 i.u./ml); the incubation was continued for an additional 30 minutes. The binding of ^{125}I -r31 kD was then determined by measurement of radioactivity by a gamma counter.

Figure 48. Binding of fibrin binding domain polypeptides to the fibrin clot

5 This experiment was carried out essentially as described for the two-step Reaction II (Example 11). 0.15 μ M 125 I of one of the fibrin binding domain polypeptides as indicated below was incubated at 37°C with preformed fibrin clot derived from 20 μ l citrated whole blood. The binding was measured in the presence of 5 mM CaCl₂ and 10 0.02 units/ml transglutaminase. The reaction was terminated, after a 45 minute incubation, by centrifugation; the pellet was washed three times with PBS and the radioactivity was measured in a gamma counter.

- 15
1. plasmatic 31 kD FBD (p31 kD)
 2. r12 kD
 3. r20 kD
 4. r31 kD (Batch A)
 - 20 5. r31 kD (Batch B)
 6. r31 kD (Batch C)

Figure 49. Comparison of binding of 125 I-r12 kD to fresh and frozen fibrin clots

25 This experiment was carried out essentially as described for the two-step Reaction II (Example 11). Preformed fibrin clots derived from 20 μ l citrated whole human blood were either frozen at -70°C for 7 days (frozen clots) or used immediately after their formation (fresh clots).

30

The fibrin clots were incubated with 0.15 μ M 125 I-r12 kD in the presence or absence of 0.02 units/ml guinea-pig

liver transglutaminase (Sigma). The binding to fibrin clots was measured as described in Example 11.

5 Figure 50. Conditions for binding of ^{125}I -r31 kD FBD to preformed clots

This experiment was carried out essentially as described for the two-step Reaction II (Example 11).

10 The fibrin clot was produced in citrated blood (A) and "naive" blood (B). The clots were incubated for the binding step at 37°C in a final volume of 250 μl PBS with 0.15 μM ^{125}I -rFBD and other constituents as indicated in the figure and below. The concentrations given are the
15 final concentration in the reaction mixture.

1. Control (no added constituents)
2. 5 mM CaCl_2
3. 5 mM CaCl_2 / 2 units/ml hirudin (Sigma)
- 20 4. 5 mM CaCl_2 / 2 units/ml hirudin/ 0.02 units/ml transglutaminase
5. 5 mM CaCl_2 / 0.02 units/ml transglutaminase
6. 0.02 units/ml transglutaminase

25 (All the exogenous transglutaminase used in the experiments described in this application was guinea-pig liver transglutaminase from Sigma).

30 Figure 51. Binding of ^{125}I -r31 kD FBD to ECM in the presence of various constituents

0.3 μM of ^{125}I -r31 kD FBD was incubated with extracellular matrix of endothelial cells (ECM) as described by Eldor et al. (20) in a 37°C CO_2 -incubator in the
35 presence of saline, 3 mM CaCl_2 and 0.02 units/ml of

guinea-pig liver transglutaminase (TG) and other constituents as indicated in the figure. The control was done in the absence of transglutaminase.

5 The reactions were terminated after 45 minutes by washing five times with PBS. Plates were extracted with a solution of 0.5% SDS and aliquots of the extracted material were measured in a gamma counter.

10 Figure 52. Refolding and purification of the r20 kD polypeptide as monitored by elution profiles from a Superose 12 column (attached to a FPLC)

15 Aliquots of 200 μ l of the r20 kD polypeptide at various stages of the refolding and purification process were injected on top of a Superose 12 column (attached to a FPLC). The column was equilibrated and eluted with a solution of 150 mM NaCl/20 mM Tris HCl, pH 7.8, at a flow rate of 0.8 ml/min. The lower trace is from the FPLC Controller LCC-500. A. Pellet of r20 kD polypeptide solubilized in 6 M Guanidine-HCl and reduced with 50 mM β -mercaptoethanol; B. Refolded and air-reoxidized r20 kD polypeptide; C. Q-Sepharose bound polypeptides, i.e., material which was separated from the purified r20 kD; D. Flow-through from the Q-Sepharose column; E. Flow-through from the Heparin-Sepharose column, i.e., material which was separated from the purified r20 kD; F. Purified 20 kD polypeptide (retention time = 18.16 min), eluted from the Heparin-Sepharose column with 0.5 M NaCl. Note that there is no peak at this retention time of 18.16 min in Profile A, where the material is in reduced form, nor in Profiles C & E, which contain incorrectly folded forms of the 20 kD polypeptide.

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Figure 53. Refolding and purification of the r12 kD polypeptide as monitored by elution profiles from a Superose 12 column (attached to a Waters HPLC system)

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Aliquots of 25-100 μ l of the r12 kD polypeptide at various stages of the refolding and purification process were injected on top of a Superose 12 column (attached to a Waters HPLC system). The column was equilibrated and eluted with a solution of 150 mM NaCl/20 mM Tris HCl, pH 7.8, at a flow rate of 0.8 ml/min. A. Pellet of r12 kD polypeptide solubilized in 6 M Guanidine-HCl and reduced with 50 mM β -mercaptoethanol; B. Refolded and air-reoxidized r12 kD polypeptide; C. Q-Sepharose bound polypeptides, i.e., material which was separated from the purified r12 kD; D. Flow-through from both the Q- and Heparin-Sepharose columns (in this case, the columns were connected in series and the flow-through from the Q-Sepharose was therefore automatically loaded on the Heparin-Sepharose column), i.e., material which was separated from the purified r12 kD; E. Purified r12 kD polypeptide (retention time - 18.83 min), eluted from the Heparin-Sepharose column with 0.5 M NaCl. Note that there is no peak at this retention time of 18.83 min in Profile A, where the material is in reduced form, nor in Profiles C & D, which contain incorrectly folded forms of the r12 kD polypeptide.

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DETAILED DESCRIPTION OF THE INVENTION

5 The plasmids pFN 975-25, pFN 949-2, pFN 137-2, and pFN
196-2 were deposited pursuant to, and in satisfaction of,
the requirements of the Budapest Treaty on the
International Recognition of the Deposit of Microor-
ganisms for the Purposes of Patent Procedure with the
American Type Culture Collection (ATCC), 12301 Parklawn
Drive, Rockville, Maryland 20852 under ATCC Accession
10 Nos. 67832, 67831, 67910, and _____, respectively.
Similarly, many of the other ATCC deposits referred to in
the subject application were also deposited pursuant to,
and in satisfaction of, the requirements of the Budapest
Treaty.

15 The fibrin binding domain of plasma fibronectin consists
of five repetitive finger-like loops of 41-52 amino acids
in length. Each loop has two adjacent intra-loop
disulfide bonds, i.e. 4 cysteine residues per loop. This
20 repeat structure is termed Type I homology (8).

The recombinant fibrin binding domain (FBD) polypeptides
described in this application comprise either the full
length fibrin binding domain (the r31 kD polypeptide) or
25 smaller proteins (r20 kD and r12 kD polypeptides) of the
fibrin binding domain. These smaller polypeptides are
smaller than 31 kD and comprise part of the sequence of
the fibrin binding domain. Many other polypeptides of
the fibrin binding domain may be expressed by additional
30 plasmids constructed, using methods known in the art,
from plasmids described in this application and these
polypeptides may be refolded, reoxidized, and purified
using methods described in this application.

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The full length recombinant fibrin binding domain (the r31 kD polypeptide) described in this application comprises the first 262 amino acids of fibronectin which the sequence arg-ala-ala-val at the carboxy-terminus. It is not yet known if an additional methionine residue is present at the amino terminus of the final polypeptide. The plasmatic fibrin binding domain derived by tryptic digestion of plasma fibronectin comprises the first 259 amino acids of fibronectin, i.e. with arginine at the carboxy-terminus.

The r31 kD polypeptide has five of the Type I homology loops discussed above (i.e. 10 disulfide bonds), the r20 kD polypeptide has three loops (i.e. 6 disulfide bonds), and the r12 kD polypeptide has two loops (i.e. 4 disulfide bonds). The presence of these disulfide bonds explains the necessity and also the difficulty of the refolding/reoxidation procedure developed to obtain and purify correctly folded FBD polypeptides which have the correct disulfide bonds. The correctly folded FBD polypeptides are biologically active, i.e. they can bind to fibrin and/or to Staphylococcus aureus.

The recombinant FBD polypeptides are produced in inclusion bodies which are contained in the pellet produced after disruption of the cell cake.

This invention discloses the production of recombinant fibronectin fibrin binding domain (FBD) polypeptides for use in thrombus imaging, prevention of thrombus formation, and prevention of bacterial infection. These polypeptides may also be bound to a thrombolytic agent for targeting the agent to a thrombus.

The recombinant cells which produce the FBD polypeptides can be any cells in which a DNA sequence encoding an FBD polypeptide has been introduced by recombinant DNA techniques. The cell must be capable of expressing the DNA sequence and producing the polypeptide product. The cell may be a mammalian cell, a fungal cell such as a yeast cell, or a bacterial cell.

The bacterial cell can be any strain including auxotrophic, prototrophic and lytic strains, F⁺ and F⁻ strains, strains harboring the cI857 repressor sequence of the λ prophage and strains deleted for the deo repressors or the deo gene.

Examples of wild type Escherichia coli strains are prototroph ATCC No. 12435, and auxotroph MC1061 (ATCC Accession No. 67361).

Examples of Escherichia coli strains which harbor the λ cI857 repressor sequence are the auxotrophs A1645 harboring plasmid pTVR 279-8 (ATCC No. 53216), A1637 harboring plasmid pTV 104(2) (ATCC No. 39384), and A2097 harboring plasmid pSOD α 2 (ATCC No. 39786), and the prototrophs A4255 harboring plasmid pFN 975-25 (ATCC No. 67832) and biotin-independent A4346 harboring plasmid pHG44 (ATCC No. 53218).

An example of a lytic Escherichia coli strain is A4048 which harbors plasmid pHG44 (ATCC No. 53217).

Examples of F⁻ strains are Escherichia coli s ϕ 930 (F⁻) harboring plasmid pMF 5534 deposited under ATCC No. 67703 and Escherichia coli W31100 (F⁻) harboring plasmid pEFF 920 deposited under ATCC No. 67706.

Examples of Escherichia coli strains deleted for the deo gene or deo repressors are SØ732 harboring plasmid pMF 2005 (ATCC No. 67362), SØ540 harboring plasmid pJBF 5401 (ATCC No. 67359), and SØ930 harboring plasmid pEFF 920 (ATCC No. 67706) (see European Patent Application Publication No. 0303972, published February 22, 1989).

The plasmids of this invention may be introduced into suitable bacterial host cells, preferably Escherichia coli. An example of a suitable Escherichia coli cell is strain A4255 (F⁺) [ATCC Accession No. 67832], but other Escherichia coli strains and other bacteria can also be used as host cells for the plasmids. Such bacteria include Pseudomonas aeruginosa and Bacillus subtilis.

All of the Escherichia coli host strains described above can be "cured" of the plasmids they harbor by methods well known in the art, e.g. the ethidium bromide methods described by R.P. Novick in Bacteriol. Review 33: 210 (1969).

The bacterial cell may contain the FBD sequence encoding the FBD polypeptide in the body of a vector DNA molecule such as a plasmid. The vector or plasmid is constructed by recombinant DNA techniques so that the sequence encoding the FBD polypeptide is incorporated at a suitable position in the molecule.

Plasmids used for production of the FBD polypeptides can harbor a variety of promoters such as the λ promoter or the deo promoters.

Among the plasmids which may be used for production of FBD polypeptides are the following:

- 5 a) Plasmid pFN 975-25 which expresses the r31 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. 67832;
- 10 b) Plasmid pFN 949-2 which expresses the r20 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. 67831;
- 15 c) Plasmid pFN 196-2 which expresses the r12 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. _____;
- 20 d) Plasmid pFN 197-10 which expresses a modified 12 kD FBD polypeptide, and which has been described in Figure 37 of this application;
- 25 e) Plasmid pFN 195-4 which expresses the r31 kD polypeptide fused to the sequence DGRGDS, and which has been described in Figure 39 of this application;
- 30 f) Any plasmid, derived from the above plasmids, containing FBD sequences encoded by the above plasmids; and
- g) Any plasmid which contains FBD sequences encoded by the above plasmids.

35 The subject invention provides an imaging agent which comprises a polypeptide labeled with an imageable marker, such polypeptide having an amino acid sequence substantially present in the fibrin binding domain of

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The detectable marker may also be a paramagnetic ion. Paramagnetic ions are also commonly used in medicine. Examples of such markers include chelated metal ions of chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), praseodymium (III), neodymium (III), samarium (III), gadolinium (III), terbium (III), dysprosium (III), holmium (III), erbium (III), ytterbium (III), or mixtures thereof.

Preferably, the imaging agent comprises a polypeptide which is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

The subject invention also provides a method for imaging a fibrin-containing substance, i.e. a thrombus or atherosclerotic plaque, which comprises contacting the fibrin-containing substance to be imaged with the agent as disclosed above under conditions such that the agent binds to the fibrin-containing substance and imaging bound agent and thereby imaging the fibrin-containing substance.

Further provided is a method for imaging a fibrin-containing substance in a subject which comprises:

(a) administering to the subject a composition of the agent as disclosed above under conditions permitting the imaging agent therein to enter the blood stream and bind to fibrin present in the blood vessels;

(b) imaging bound agent within the blood vessels; and thereby

(c) imaging the fibrin-containing substance.

Preferably, the polypeptide of the reagent used in the above methods for imaging a fibrin-containing substance is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

Preferred markers used in the above methods for imaging a fibrin-containing substance are radioactive isotopes, elements which are opaque to X-rays, or paramagnetic ions. Most preferred markers are radioactive isotopes,

such as indium-111, technetium-99m, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-133, and gallium-67.

Imaging may be done through any of the methods known to one skilled in the art. These methods include but are not limited to X-ray, CAT scan, PET scan, NMRI, and fluoroscopy. Preferably, the imaging of the fibrin-containing substance by the above methods is carried out using a gamma camera.

Further provided is a plasmid for expression of a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

Applicants have provided four examples of fibrin binding domain polypeptides. These include the p31 kD, r31 kD, r20 kD, and r12 kD polypeptides. These polypeptides exhibit the binding and adhesive properties of portions of naturally-occurring human fibronectin. The scope of the claims of the subject application are not intended to be limited to these four FBD polypeptides, which are examples of preferred embodiments only.

Three forms of the r31 kD fibrin binding domain polypeptide are defined in Example 5 as:

- (a) the polypeptide as it is obtained from a washed pellet, after dissolution in 6 M GuCl, i.e. in "scrambled" form;

(b) the fully reduced polypeptide, present after treatment with a reducing agent such as GSH in the presence of 6 M GuCl; and

5 (c) the reoxidized-refolded polypeptide, obtained by treatment with the GSH/GSSG as described in Example 5.

10 The "scrambled" r31 kD polypeptide is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

15 Unless otherwise stated, the r31 kD polypeptide described is correctly folded, i.e. form (c). Similarly, the 20 kD and 12 kD polypeptides may also occur in these three forms but the 20 kD and 12 kD polypeptides used in the experiments are correctly folded.

20 In preferred embodiments, the polypeptide is about a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin; about a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin; or about a 12 kD polypeptide
25 corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin.

30 In more preferred embodiments, the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding
35 domain of human fibronectin and having the amino acid

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sequence of amino acids 1-153 as shown in Figure 1; the
20 kD polypeptide comprising less than about 20
additional amino acids; or a 12 kD polypeptide
corresponding to an amino acid sequence present in the
fibrin binding domain of human fibronectin and having the
amino acid sequence of amino acids 1-109 as shown in
Figure 1.

Naturally-occurring human fibronectin is as it occurs in
the human body (in plasma).

As used throughout this application, a substantial
portion is at least one fifth (1/5). A polypeptide which
has the biological activity of the fibrin binding domain
of naturally-occurring human fibronectin exhibits binding
or adhesive properties similar to the fibrin binding
domain of naturally-occurring human fibronectin when the
level of such activity is assayed or determined.

In this invention, the amino acid sequence of the various
functional domains are determined by cleavage of the cDNA
which encodes the domains with restriction enzymes, and
do not necessarily correspond to the amino acid sequence
of the domains as obtained and defined by proteolytic
digestion of fibronectin.

The plasmid of this invention further comprises suitable
regulatory elements positioned relative to the DNA
encoding the polypeptide so as to effect expression of
the polypeptide in a suitable host cell, such as pro-
motors and operators, e.g. λ P₁O₁, ribosomal binding
sites, e.g. C₁₁, and repressors. Other suitable regu-
latory elements include, for example, the lac, trp, tac,
lpp and deo promoters (European Patent Application
Publication No. 0303972, published February 22, 1989).

The suitable regulatory elements are positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable bacterial host cell. In preferred embodiments of the invention, the regulatory elements are positioned close to and upstream of the DNA encoding the polypeptide.

The invention provides a plasmid designated pFN 975-25 and deposited in Escherichia coli strain A4255 (F⁺) under ATCC Accession No. 67832. Plasmid pFN 975-25 encodes a 31 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-262.

Further provided is a plasmid designated pFN 949-2 and deposited in Escherichia coli strain A1645 under ATCC Accession No. 67831. Plasmid pFN 949-2 encodes a 20 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-153 and less than 20 additional amino acids.

Also provided is a plasmid designated pFN 196-2 and deposited in Escherichia coli strain A4255 under ATCC Accession No. _____. Plasmid pFN 196-2 encodes a 12 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-109.

In presently preferred embodiments, the invention provides an Escherichia coli cell containing the plasmid designated pFN 975-25 and wherein the cell is deposited under ATCC Accession No. 67832; an Escherichia coli cell containing the plasmid designated pFN 949-2 and wherein the cell is deposited under ATCC Accession No. 67831; and an Escherichia coli cell containing the plasmid designated pFN 196-2 and wherein the cell is deposited under ATCC Accession No. _____.

The invention provides a method of producing a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises treating a cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and recovering from the cell the polypeptide so expressed.

Preferably, the polypeptide so produced is a 31 kD, 20 kD, or 12 kD polypeptide of the fibrin binding domain.

Further provided is a purified polypeptide substantially free of other substances of human origin which has an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

Preferably, the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

The invention further provides such a purified polypeptide substantially free of other substances of

human origin fused to a second polypeptide, the second polypeptide comprising a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin.

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Preferably, the fused polypeptide is a 45 kD fused polypeptide, wherein the purified polypeptide is a 12 kD polypeptide and the second polypeptide which comprises a substantial portion of the cell binding domain of naturally-occurring human fibronectin is a 33 kD polypeptide. The fused polypeptide may also comprise a 31 kD purified polypeptide and a second polypeptide which contains the amino acid sequence GRGDS. Another preferred fused polypeptide is a 64 kD fused polypeptide, wherein the purified polypeptide is a 31 kD polypeptide and the second polypeptide which comprises a substantial portion of the cell binding domain of naturally-occurring human fibronectin is a 33 kD polypeptide.

The invention also provides a plasmid for expression of the 45 kD fused polypeptide, disclosed above, designated pFN 202-5; a plasmid for expression of the 31 kD/GRGDS fused polypeptide, disclosed above, designated pFN 195-4; and a plasmid for expression of the 64 kD fused polypeptide, disclosed above, designated pFN 194-2.

As used throughout the subject application, "fused" or "bound" encompasses polypeptides bound covalently, non-covalently, or conjugated. The polypeptides may be conjugated through other chemical moieties including amino acid or polypeptide cross-linkers, which are standardly used in the art and are well-known to those skilled in the art to which the subject invention pertains.

This invention further provides a method of treating a subject susceptible to, or afflicted with, a bacterial infection which comprises administering to the subject an amount of any of the disclosed polypeptides effective to prevent or treat the bacterial infection. The susceptibility to bacterial infection may be due to the presence of a catheter or an implant in the subject.

Numerous methods are known in the art for detection of thrombi, such as radioactive labeling (nuclear medicine use of isotopes), radio-opaque labeling (such as CAT scan), and Magnetic Resonance Imaging (MRI). Any of these labeling methods can be used in the method of the subject invention for detecting the thrombus. In each of these detection methods the polypeptide is used as a diagnostic agent for detecting the thrombus.

The invention provides a coated medical device comprising a medical device and the polypeptides of the fibrin binding domain of naturally-occurring human fibronectin applied as a coating to the surface of the medical device. Examples of medical devices which may be coated include catheters, medical implants (such a hip replacement and prostheses), tubings and syringes.

The invention provides a method of minimizing risk of bacterial infection associated with use of medical devices, preferably a catheter, which comprises:

- (a) applying the polypeptide of the fibrin binding domain of fibronectin as a coating to a surface of the device; and
- (b) employing the resulting coated device rather than an uncoated device.

The invention also provides a method of minimizing risk of bacterial infection associated with the use of medical devices which comprises employing a device coated with the polypeptides disclosed in the subject application rather than an uncoated device.

Also provided is a method of refolding and reoxidizing a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide.

Preferably, the thiol-containing compound is selected from the group consisting of glutathione, thioredoxin, β -mercaptoethanol, and cysteine.

Preferably, the thiol-containing compound is β -mercaptoethanol and the disulfide is produced in situ by introduction of air.

Preferably, the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.

The method of refolding and reoxidizing may additionally comprise contacting the polypeptide with a denaturant. Preferred denaturants are guanidine hydrochloride and urea.

Preferably, the polypeptide is at a low concentration, such as below 600 $\mu\text{g/ml}$.

The subject invention also provides a method for recovering a purified biologically active polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin from a cell in which the polypeptide has been produced by means of expression of a plasmid containing DNA encoding the polypeptide which comprises:

- (a) disrupting the cell so as to produce a lysate containing the polypeptide;
- (b) centrifuging the lysate so as to concentrate the polypeptide;
- (c) separating the concentrated polypeptide;
- (d) solubilizing the separated, concentrated polypeptide;
- (e) refolding and reoxidizing the solubilized polypeptide;
- (f) separating the refolded and reoxidized polypeptide; and
- (g) recovering the purified, refolded and reoxidized polypeptide.

Preferably, the refolding and reoxidizing comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide. Preferably, the thiol-containing compound is selected from the group consisting of

glutathione, thioredoxin, β -mercaptoethanol, and cysteine.

5 In one preferred embodiment, the thiol-containing compound is β -mercaptoethanol and the disulfide is produced in situ by introduction of air.

10 Preferably, the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.

15 The method may additionally comprises contacting the polypeptide with a denaturant, such as guanidine hydrochloride or urea.

20 Preferably, the polypeptide is at a low concentration, such as below 600 μ g/ml.

25 Preferably, the separating of the concentrated polypeptide in step (c) comprises chromatography, preferably Heparin-Sepharose chromatography.

30 The subject invention also provides a method of inhibiting thrombus formation in a subject susceptible to thrombus formation which comprises administering to the subject an amount of a polypeptide (selected from the polypeptides and fused polypeptides disclosed above) effective to inhibit thrombus formation. The polypeptide may be reduced or alternatively the S-H groups may be blocked (e.g. by carboxyamidation to prevent reoxidation).

35 The subject invention also provides a polypeptide as disclosed above bound to a thrombolytic agent for targeting of thrombolytic agents. The thrombolytic

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EXAMPLES

5 All the references to map positions correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA shown in Figure 1 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

10 This patent application is directed to polypeptides of the N-terminus fibrin binding domain (FBD). Some of the proteins described are fusion proteins comprising an FBD fragment joined at its C-terminus to a fragment of the cell binding domain (CBD).

15 The cDNA sequence corresponding to the CBD which applicants have cloned and expressed is missing the 270 bp extra domain (ED) segment which extends from nucleotides 4812 to 5081, inclusive, on the Baralle map (see Figure 1). Thus, the cDNA sequence which is said to extend from
20 nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because it is missing the 270 nucleotides of the ED segment, namely from nucleotides 4812 to 5081 inclusive; this region is also known in the art as the ED-A region; concomitantly amino acid 1690 is changed
25 from alanine to threonine. Similarly, the polypeptide expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on the Baralle map but would be missing the 90 amino acids encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would
30 contain only 660 amino acids. This is true for all CBD fragments described in this application which span the ED region. (The region known in the art as the ED-B region is missing both in Baralle's sequence and in applicants' cDNA.)

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EXAMPLE 1

Preparation of a Fibronectin cDNA Library

A cDNA library was prepared in λ gt11 from poly A⁺ mRNA isolated from human liver according to the published procedures (13,14). The cDNA fragments were cloned using EcoRI linkers and the cDNA library was screened for fibronectin (FN) positive plasmids using the following synthetic DNA probes.

Probes for cell binding domain (CBD):

<u>Probe</u>	<u>Nucleotides</u>
(3') CACTCTATAATGTCCTAGTGAATGCCTCTTTGTCCTCC	(4355-4392)
(3') AGAATCTCCTTCTGTCTTTTGTCCAGAACTAAG	(3967-3999)
(3') CCGGTTGTTAGTTGTCAAAGACTACAAGGCTCCCTGGACC	(4200-4239)

Probes for N-terminal fibrin binding domain (FBD):

(3') GGGGGTCCGAGGGATACCGGTGACACAGTGTCTTAA	(817-850)
(3') CGACGGGTGCTCCTTTAGACGTGTTGGTTACTTCCCCAGTAC	(1310-1340)

A series of FN cDNA clones covering the entire region of fibrin, collagen, heparin and cell binding domains was identified and isolated (Figure 32). The cDNA fragments were subcloned into the EcoRI site of pBR322.

The mRNA of FN is alternatively spliced and therefore different length cDNA's have been reported in the literature. Applicants' cDNA corresponding to the cell binding domain has a 270 base pair deletion from base 4811 to base 5080 on the FN physical map (the complete non spliced cDNA).

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EXAMPLE 2

Expression and Purification of Fibrin Binding Domain
(FBD) Polypeptides

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A. Expression of a partial FBD 20 kD polypeptide

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The FN cDNA clones obtained as described in Example 1 and depicted in Figure 32, did not include DNA encoding amino acids 1-190 of the FN molecule. These amino acids are part of the FBD. The DNA corresponding to nucleotides 14 to 472 and coding for amino acids 1-153 (Figure 1A) was constructed by ligation of 7 pairs of chemically synthesized nucleotides (Figures 2 and 3). The synthetic DNA fragment was designed to contain an ATG initiation codon at the 5' end as well as convenient restriction sites for introduction into various expression vectors. To enable further manipulation of the DNA sequence coding for the FBD, nucleotide number 19, thymidine (T) was changed to adenine (A), thereby eliminating a DdeI restriction site without altering the amino acid sequence. (The site of the nucleotide change is denoted by an asterisk in linker #1 shown in Figure 2A.) The various steps for the cloning of the above synthetic DNA fragment into pBR322 - plasmid vector digested with EcoRI and BamHI are described in Figure 3. The plasmid obtained was designated pFN 932-18. The DNA fragment coding for the first 153 N-terminal amino acids of fibronectin from plasmid pFN 932-18, was inserted into pTV 301, a λ P_t expression vector, between the NdeI and BglII sites replacing the DNA sequence coding for human growth hormone (hGH) in plasmid pTV 301 (Figure 4).

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The resulting plasmid, pFN 949-2, was deposited with the American Type Culture Collection under Accession No.

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67831. Plasmid pFN 949-2 was used to transform Esche-
richia coli prototroph A4255. These transformed Esche-
richia coli cells were found to express the partial FBD
polypeptide in amounts comprising about 5% of the total
cellular proteins. The polypeptide has a mobility of
about 20 kD on reduced SDS polyacrylamide gels as
determined from the mobility of the size markers. The
polypeptide comprises the first 153 amino acids of fibro-
nectin followed by 4 amino acids coded for by a synthetic
linker and then several amino acids resulting from
readthrough into the pBR322 vector, i.e., a total of 153
amino acids plus less than 20 additional amino acids, if
the additional N-terminal methionine is present.
Throughout this specification the polypeptide is referred
to as the r20 kD polypeptide or the r20 kD FBD.

B. Expression of a "complete" FBD polypeptide

In order to obtain expression of the entire FBD
polypeptide containing amino acids 1 to 262 the following
plasmids were constructed:

1. Insertion of termination codon TAA at the 3'
end

A synthetic oligonucleotide containing a TAA termination
codon and a BglII site having the following sequence:

5' CTGTTTAAATAAGCA
3' GACAAATTCGTCTAG

was ligated to the 3' end of an EcoRI-PvuII fragment
isolated from FN cDNA clone p931-5 and to a pBR322 vector
digested with EcoRI and BamHI as described in Figure 5.
The plasmid obtained was designated pFN935-12.

2. Subcloning of carboxy terminal region of FBD
in a λ P₁ expression vector

An EcoRI-HincII DNA fragment coding for the carboxy
terminal region of the FBD was isolated from plasmid
pFN935-12 and ligated to plasmid pTV 194-80 digested with
EcoRI and SmaI as described in Figure 6. The plasmid
obtained was designated pFN 946-12.

3. Syntheses and cloning of DNA corresponding to
nucleotides 468-599 of FN

Three pairs of chemically synthesized nucleotides were
ligated to an EcoRI-DdeI FN fragment isolated from
plasmid pFN932-18 (Figure 3) in the presence of pUC19
vector DNA (purchased from GIBCO BRL Co.) digested with
EcoRI and XbaI as described in detail in Figure 7. The
plasmid obtained was designated pFN 948-4.

4. Construction of a plasmid encoding the com-
plete FBD region

In order to construct a plasmid which codes for the
entire FBD, amino acid 1 to amino acid 262, an EcoRI-XbaI
DNA fragment coding for FN was isolated from plasmid
pFN948-4 and inserted into plasmid pFN 946-12 digested
with EcoRI and XbaI as described in Figure 8. The
plasmid obtained was designated pFN-957. This plasmid
contains the complete coding sequence for FBD but does
not express the FBD polypeptide as it lacks a ribosomal
binding site (RBS).

5. Expression of the FBD under λ P_L promoter and cII RBS

5 An NdeI-HindIII fragment containing the FBD coding region and the T₁T₂ transcription terminators was isolated from plasmid pFN-957 and inserted into plasmid pTV 301 (Figure 33) digested with NdeI and HindIII as described in Figure 9. The resulting plasmid, designated as pFN 962-3, directs the expression of a FBD polypeptide under the control of λ P_L promoter and cII ribosomal binding site. Escherichia coli strains A1645 and A4255 transformed with this plasmid expressed only small amounts of the FBD polypeptide. The expression of the FBD polypeptide was detectable only by Western blot analysis using polyclonal antibodies directed against human plasma derived FN.

6. Expression of an FBD polypeptide under the λ P_L promoter and the β -lactamase promoter and ribosomal binding site

As the level of expression of the FBD polypeptide obtained with plasmid pFN 962-3 was low, we added a DNA fragment coding for the β -lactamase promoter and β -lactamase RBS (PBLA). The DNA fragment coding for PBLA was isolated from plasmid pBLA11 (ATCC Accession No. 39788) and inserted into plasmid pFN 962-3 digested with NdeI, filled in with Klenow enzyme and digested with EcoRI as described in Figure 10. The plasmid obtained, designated pFN 975-25, was deposited with the American Type Culture Collection under ATCC Accession No. 67832. This plasmid was used to transform Escherichia coli prototroph A4255 (F+).

These Escherichia coli cells were found to express the "complete" FBD polypeptide at levels comprising about 5-8% of the total cellular proteins. The polypeptide migrated on SDS-PAGE gels with an apparent molecular weight of 31 kD, hence it is referred to as the 31 kD polypeptide or the r31 kD FBD.

C. Fermentation and growth conditions

The clone expressing the r31 kD FBD polypeptide was fermented in rich medium (yeast extract and casein hydrolysate) containing ampicillin. Growth was carried out at 30°C. Expression was obtained upon induction at 42°C for 2 hours, and subsequently bacterial cell cake containing the r31 kD FBD polypeptide was obtained. Similarly, the clone expressing the r20 kD FBD was fermented and bacterial cell cake containing the r20 kD FBD polypeptide was obtained.

D. Refolding and purification of recombinant fibrin binding domain (r31 kD) polypeptide

The process is made up of three stages:

1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

1. Crude processing

The cake is disrupted first in 5 volumes of 50 mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1); the pellet is then treated with 1.2 volumes of Buffer 1 containing 100 mg/liter lysozyme (2 hours agitation at 37°C). Triton X 100 is added to the resulting suspension (to 1%), and

after 30 min. at room temperature the suspension is centrifuged and the pellet is resuspended and washed twice with water. All these steps are performed by disruption of the pellet and centrifugation and the 31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2 mM PMSF/2mM 6-aminocaproate, pH 7.5 (Buffer A) and then treated successively with Buffer A containing: 1% decyl sulfate, 1% decyl sulfate/5% glycerol and 5% glycerol. The final treatment is with Buffer A without additives.

2. Refolding/reoxidation

Principle: To dissolve the pellet in 6M guanidine-HCl - GuCl - in the presence of a thiol reducing agent, such as glutathione - GSH - and to refold/reoxidize at a lower GuCl concentration by the addition of oxidized glutathione-GSSG.

The washed pellet from step 1 above is dissolved in 150-700 volumes of 6M GuCl/3mM GSH in Buffer A. The concentration of GuCl is lowered gradually, i.e., first 2 M, then 1 M and 0.5 M, while keeping the concentration of all other components constant, except for the volume, which at this stage is brought to 500-1000 fold higher than that of the pellet. At one of the intermediate concentrations of GuCl, i.e., between 0.5 and 2 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at room temperature for 24-48 hours. The refolded 31 kD is then dialyzed against Buffer A without additives.

3. Purification

Concentration: The large volume of refolded 31 kD is first centrifuged to remove the insoluble pellet that contains no 31 kD and is then dialyzed against Tris-HCl, pH 7.8, before being concentrated and initially purified on a Heparin-Sepharose column.

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10

EXAMPLE 3

Bacterial Binding Activity

5 Experiments have been performed on the binding of r31 kD
FBD to bacterial suspensions of Staphylococcus aureus.
Identical binding curves were obtained for radio-iodinat-
ed intact plasma fibronectin, the proteolytic 31 kD amino
10 terminal fragment (p31 kD) derived from human plasma
fibronectin, and r31 kD FBD.

The inhibition of bacterial binding by [¹²⁵I] p31 kD
indicated that the recombinant 31 kD FBD competes with
the authentic proteolytic fragment.

15 The bacterial binding activity of the r31 kD FBD is
described in more detail in Example 7, Section II:
Bacterial Binding Activity.

20

EXAMPLE 4

Inhibition of Bacteria Adhesion

- 5 To estimate the capacity of r31 kD FBD to interfere with
the adherence of bacteria to the extracellular matrix in
wounds, a competition assay was developed. In this
assay, adherence of Staphylococcus aureus to a plastic
surface coated with fibronectin and the interference of
10 FBD with adherence were measured. Both authentic FBD and
r31 kD FBD were active in inhibiting bacterial adhesion
to the fibronectin coated surface.

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EXAMPLE 5

Refolding and Purification of Recombinant 31 kD Fibrin-Binding Polypeptide of Fibronectin

5

The following is an improved procedure for the purification of the recombinant 31 kD fibrin-binding domain polypeptide (r31 kD) produced as described in Example 2.

10

The process is made up of three stages:

1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

15

1. Crude processing

20

1.1 Washing of the pellet: The bacterial cell cake is disrupted first in 5 volumes of 50 mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1). The pellet is then successively treated with Buffer 1 containing 100 mg/liter lysozyme (2 hours at 37°C), Buffer 1 containing 1% Triton X-100 (30 minutes at room temperature) and twice with water. All these steps are performed by disruption of the pellet and centrifugation; the r31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

25

30

1.2 Extraction of the pellet: The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2 mM PMSF/2 mM epsilon-aminocaproate (Buffer A) pH 7.5, and then treated successively with Buffer A containing: (a) 1% decyl sulfate; (b) 1% decyl sulfate/5% glycerol; and (c) 5% glycerol.

The final treatment is with Buffer A without additives.

2. Solubilization and Refolding/reoxidation

A refolding/reoxidation procedure for the recombinant 31 kD polypeptide (r31 kD) has been developed and refined.

2.1 Principle: To dissolve the pellet in 6 M guanidine-HCl (GuCl) in the presence of a thiol reducing agent, such as glutathione (GSH) and to refold/reoxidize at a lower GuCl concentration by the addition of oxidized glutathione (GSSG).

2.2 Procedure: The extracted pellet is dissolved in 100-700 volumes of 6 M GuCl/3 mM GSH in Buffer A, pH 8.0. The concentration of GuCl in the dialysis buffer is lowered gradually, e.g., first 3 M, then 1.5 M and finally 0.5 M, while keeping the concentration of all other components constant. At one of the intermediate concentrations of GuCl, i.e., between 2 M and 1 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at pH 8 at room temperature for 48-72 hours. The refolded r31 kD is then dialyzed against Buffer A at pH 8.5, without additives.

Example: Approximately 10 grams of extracted pellet (see 1.2) were homogenized and dissolved in 1 liter of Buffer A/6 M GuCl/3 mM GSH/pH 8 and the suspension was stirred for 14 hours until it was a clear solution. This solution was dialyzed for 24 hours against four liters of Buffer A which additionally contained 3 mM GSH and 3 M GuCl, pH 8. Subsequently, the resulting solution was dialyzed

for 24 hours against 8 liters of Buffer A containing 3 mM GSH, pH 8. The resulting solution was dialyzed twice against 10 liters of Buffer A containing 0.3 mM GSH and 0.3 mM GSSG, pH 8. The process of dialysis, during which reoxidation also occurred, lasted approximately 80 hours. Finally, the glutathione was removed from the refolded protein by dialysis against 10 liters of Buffer A, pH 8.3 - 8.5. This step was performed twice. Subsequently, the solution was loaded on a phenyl-Sepharose column.

2.3 Alternative procedure: Similar results have been obtained when cysteine (3 mM) was used instead of glutathione and cystine (0.3 mM) instead of oxidized glutathione.

2.4 Use of thioredoxin: Attempts were also made to increase the rate of reoxidation of the r31 kD, by using thioredoxin. Based on SDS-PAGE profiles run in the absence of mercaptoethanol (ME), thioredoxin reduction and reoxidation of "scrambled" material seems to yield a more homogeneous preparation of r31 kD, but the concentration of thioredoxin which had to be used was about 100 μ M. "Scrambled material" is r31 kD polypeptide which is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

3. Purification

3.1 Phenyl-Sepharose chromatography: The large volume of refolded r31 kD is first centrifuged to remove the insoluble pellet which contains either "scrambled" r31 kD or contaminants. The supernatant is

brought to 0.2 M ammonium sulfate in Buffer A and loaded onto a phenyl-Sepharose column equilibrated with Buffer A containing the same ammonium sulfate concentration. The r31 kD polypeptide is then purified by lowering the salt concentration, i.e., by elution in Buffer A.

Example: After reoxidation of the crude protein mixture, extracted from a 10 gram pellet, the suspension of refolded and "scrambled" r31 kD, as well as insoluble contaminants, is subjected to centrifugation at 13,000 rpm (17,000 x g) in a high-speed Beckman centrifuge equipped with a J-14 rotor. The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A containing 0.2 M AS. The column was washed with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl (Figures 11 and 12).

3.2 Heparin-Sepharose and ion-exchange chromatographies: The final step of purification of the r31 kD polypeptide is chromatography on Q-Sepharose from which it elutes in the flow-through fraction or on Heparin-Sepharose from which it is eluted by using a salt gradient. It also binds to S-Sepharose, but the eluted material is still contaminated with most of the impurities.

Example: Approximately 1/2 of the Buffer A peak was concentrated and purified on a 10 ml Heparin-Sepharose column, from which it was eluted by a solution of 0.5 M NaCl in Buffer A (Figure 13). The concentrated 31 kD was dialyzed against Buffer

A, pH 8.5, before being loaded on a 40 ml column of Q-Sepharose, which had previously been equilibrated with the same buffer. The purified r31 kD polypeptides, which eluted in the flow-through and wash fractions were concentrated by lyophilization, before being characterized. The column was washed free of the contaminant proteins by a step of 1 M NaCl (Figure 14). The purified material is greater than 95% pure (Figure 11).

3.3 Re-extraction of the pellet: After the refolding procedure the pellet was re-extracted and treated as above, since it still contained more than 50% of the r31 kD polypeptide probably in "scrambled" form. The total yield (including the re-extraction step) of the process, after 3 columns, was about 10% (Table A).

3.4 Characterization: The r31 kD polypeptide has been characterized and compared to its plasma derived counterpart in terms of its purity (purity profile on reduced gels of SDS-PAGE), migration position in non-reduced gels of SDS-PAGE (Figure 11), apparent molecular weight (approximately 37 kD) on Superose 12 (Figure 15), immunoblot and behavior on Heparin-Sepharose (the NaCl concentration for elution of both materials from Heparin-Sepharose was found to be approximately 0.32 M). In all of these assays the r31 kD polypeptide is similar to plasma derived fibrin binding domain.

4. Comparison between various forms of the r31 kD in terms of their reoxidation-refolding

5 Three different forms of the r31 kD polypeptide have been defined:

10 Form a: The polypeptide as it is obtained from the washed pellet, after dissolution in 6 M GuCl, i.e., in "scrambled" form.

15 Form b: The fully reduced polypeptide, present after treatment with a reducing agent such as GSH in the presence of 6 M GuCl.

Form c: The reoxidized-refolded polypeptide, obtained by treatment with the GSH/GSSG as described above.

20 These three forms can be distinguished from one another by:

25 (i) Their migration on SDS-PAGE gels in the absence of reducing reagents (i.e., without ME), and in the presence of the thiol-trapping agent iodoacetamide (Figure 11); and

30 (ii) Their reaction with anti-plasmatic 31 kD on immunoblots of gels run in the absence of ME. Only the correctly reoxidized-refolded form reacted with the antibody.

35 The work described above on reoxidation in the presence of GSH/GSSG (or in the presence of cysteine/cystine) has shown that the r31 kD polypeptide refolds with time to

form (c) which is indistinguishable from that of the plasma derived fibrin binding domain.

Characterization of the "scrambled" r31 kD polypeptide:

5 The polypeptide seems to be less soluble in this form, as evidenced from the large amounts remaining in the pellet after extraction.

10 Also the scrambled polypeptide differs from the refolded polypeptide in its binding characteristics to both phenyl-Sepharose and Q-Sepharose.

Characterization of the fully reduced r31 kD polypeptide:

15 This polypeptide form is both more soluble and more homogeneous than the "scrambled" one. After solubilization in 6 M GuCl in the presence of GSH (or DTT or cysteine), and then lowering the concentration of denaturant to almost zero, the "reduced" polypeptide can be purified on phenyl-Sepharose in the presence of GSH.
20 Finally, the polypeptide is "reoxidized" with GSSG at 1/10 of the concentration of GSH, i.e., 0.3 mM. This did not yield refolded 31 kD, but a form of "scrambled" polypeptide different from that described in the previous paragraph, probably because the reduced polypeptide
25 slowly autooxidizes to a scrambled form, even before it is exposed to GSSG.

5. Preparation of reduced-carboxamidated 31 kD polypeptide

30

Purified plasma derived or recombinant 31 kD polypeptide (approximately 0.6 mg/ml) were reduced in 4.3 M Guanidinium Hydrochloride (GuCl), 40 mM β -mercaptoethanol (ME), in 10 mM Tris-HCl, pH 8.5 for 24 hours at room
35 temperature.

Carboxamidation was achieved by adding iodoacetamide to the polypeptide in four-fold excess over the concentration of ME and the solution incubated for 1 hour at room temperature. Subsequently the GuCl concentration was reduced by gradual dialysis (to 3M, 2M, 1M and 0.5M GuCl), before being dialyzed against Buffer A. The precipitate formed was centrifuged and the concentration of the resulting 31 kD polypeptide in the supernatants were 0.34 and 0.19 mg/ml for the plasma derived and recombinant 31 kD, respectively. The reduced carboxyamidated polypeptide is also termed a reduced/blocked polypeptide because the S-H groups are blocked to prevent reoxidation.

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Table A

Purification of rilkD

STEP	VOLUME (ml)	PROTEIN CONC. (mg/ml)	TOTAL PROTEIN (mg)	PURITY (%)	AMOUNT 31kD (mg)	YIELD (%)	PURIF. DEGREE
Extracted pellet			~2000	35	~700	100	1
Refolding	1280	0.332	425	70	295	42	2.0
Phenyl-S.	220	0.96	123	85	105	15	2.4
"(1/2)			61		52		
Heparin-S.	16	2.19	35	90	32	9	2.6
Q-Sepharose	100		23 ^a	>95	22	6	>2.7
Re-extracted pellet			234			23	
Phenyl-S.			96			14	

¹ Purity determined from SDS-PAGE gels (+MB)

^a Measured after lyophilisation.

EXAMPLE 6

Pharmacodynamics of the r31 kD, r20 kD and r12 kD Fibrin Binding Domain Polypeptides

5

The intensity and resolution of a clot (thrombus) image is governed by the interplay of the rate of incorporation of the radiopharmaceutical and its blood clearance rate. In order to elucidate the metabolic behavior of the r31 kD fibrin-binding domain, and to compare it to fibronectin (FN), the r31 kD fibrin binding domain and plasma fibronectin were both iodinated with ^{125}I by the ICl method (24) and injected intravenously into rats. The results are shown in Figure 16 which represents the pharmacokinetic behavior of ^{125}I -r31 kD FBD and ^{125}I -FN. Blood samples were withdrawn at the times shown on the graph.

10

15

20

Figure 16 demonstrates that the clearance rates of the two radioactive molecules are different and after 5 hours, only 3% of the r31 kD FBD but 20% of FN respectively remain in circulation.

25

30

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Some of the rats were kept in individual metabolic cages, and accumulated urine and feces were collected at 7 hours and 24 hours. About 30% of the injected ^{125}I -r31 kD radioactivity was excreted in the urine during the first 7 hours, and more than 90% was excreted after 24 hours. All of the urinary radioactivity was trichloroacetic acid-soluble, which is indicative of proteolytic degradation. The analysis of a variety of organs (kidney, stomach, liver, lung, uterus, ovary, adrenal, colon, ileum, skin, brain, eye, muscle, bladder, heart, spleen, trachea, aorta and vena-cava) did not reveal any specific accumulation, and the kinetics of disappearance of the

radioactivity followed a pattern similar to that of the blood. In most of the organs, the specific radioactivity (cpm/gram tissue) was lower than that of the serum.

5 The results indicate that exogenous recombinant 31 kD
amino-terminal polypeptide of FN is moderately degraded
and excreted in the body. The pharmacokinetic behavior
is not consistent with a first-order kinetics, which may
10 indicate that the polypeptide is moderately distributed
in the tissues and body compartments other than blood.
This is also evident from the finding that the degree of
degradation does not increase during the 4-24 hour
period, thus reflecting a gradual release of the
polypeptide from body compartments. The exclusive and
15 relatively early appearance of the metabolites in the
urine indicates that the polypeptide is readily excreted
through the kidneys. The lack of accumulation of the
material in the liver may be an indication that this
organ is not a major locus of degradation and is not
20 involved in detoxification.

The relatively short half-life of r31 kD FBD is important
for its possible use in diagnostic imaging of thrombi.
The recombinant 31 kD FBD (r31 kD) may be labeled
25 radioactively or by other means and then introduced into
the blood for the purpose of imaging thrombi.

The shorter half-life of the molecule is also important
when utilizing it to prevent clot formation. By con-
30 trast, heparin, the current therapeutic agent of choice,
suffers from a very long half-life.

A similar experiment was performed using iodinated 31 kD
fibrin binding domain of plasmatc fibronectin and

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similar pharmacokinetics and distribution of radioactivity were observed.

5 The 31 kD polypeptide was obtained by cleavage of
plasmatic FN as follows: the plasmatic FN was purified
on a Gelatin-Sepharose column from which it was eluted
and stored in 1 M guanidinium hydrochloride. Thereafter,
206 mg of FN, after dialysis against 10 mM of Tris-HCl,
10 were digested with 0.01% of TPCK-trypsin at 37°C for 5
minutes. The tryptic digest was loaded on a DE52 column
(6 ml) and 1/5 of the flow-through fraction (50 ml) was
applied to a CM-Sepharose column (3 ml) and eluted with
a NaCl gradient (0-0.5 M). About 80% of the polypeptide
15 was recovered in the salt gradient (peak at about 220 mM)
and after dialysis to remove the salt about 1/2 of the
polypeptide was loaded on a Heparin-Sepharose column (1.5
ml) and eluted with 0.5 M NaCl. Approximately 75% of the
polypeptide was recovered in this fraction, i.e., about
1 mg (about 40% of the theoretical yield). This fraction
20 was >90% pure 31 kD polypeptide, and was iodinated by the
method described above.

Note that plasmatic 31 kD FBD contains the first 259
amino acids of FN, whereas the recombinant 31 kD FBD
25 contains the first 262 amino acids of FN.

Pharmacokinetics of the r20 kD and r12 kD fibrin binding
domain polypeptides

30 Similar experiments were performed using labeled r20 kD
and r12 kD fibrin binding domain polypeptides produced as
described in Examples 2, 9 and 10. The pharmacokinetics
of these polypeptides was found to be very similar to
that of the r31 kD polypeptide.

EXAMPLE 7

Biological activity of the recombinant Fibrin Binding Domain (r31 kD)

5

The biological activity of the purified recombinant 31 kD FBD polypeptide was compared to biological activity of either human plasma derived FN or a 31 kD FBD polypeptide derived from partial tryptic digest (Example 6) of human plasma derived FN. The biological activities assayed were binding to fibrin clot in vivo and in vitro, binding to bacteria (Staphylococcus aureus) and binding to extracellular matrix.

10

15

I. Fibrin binding activity

20

Two sets of experiments were carried out. In the first set, the binding of ^{125}I -r31 kD to fibrin was monitored during clot formation (Reaction I), while in the second set of experiments, the binding of ^{125}I -r31 kD to fibrin was monitored at various time periods after clot formation (Reaction II). Thrombin or Ca^{++} were added to the mixtures in order to enable clot formation in citrated blood.

25

Materials and Methods

Fibronectin Fibrin Binding Domain: FBD

30

A. Binding of FBD to fibrin:

Plasma derived 31 kD FBD was derived by partial tryptic digest of human fibronectin (see Example 6).

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Production of r31 kD FBD in Escherichia coli and its subsequent purification from the pellet of cell lysate was performed according to Example 5. Fibronectin was obtained from human plasma. ¹²⁵I-labeling of FBD and FN was carried out by the ICl method (24). The labeled polypeptides having specific activity of 20-200 cpm/ng, were stored at -20°C in small aliquots in a solution of 0.1% BSA-PBS and used within 2 weeks.

1. Binding of ¹²⁵I-FBD to fibrin clot during its formation (Reaction I):

The complete reaction mixture in siliconized microfuge tubes contained in a final volume of 250 µl the following components:

20-200 µl human whole blood (fresh, non-citrated, or 1-7 days old, citrated, as indicated in the figure legends)

0.1% BSA
5 mM CaCl₂
1U/ml Thrombin
¹²⁵I-r31 kD FBD

When binding was measured using non-citrated blood, CaCl₂ and thrombin was not added ("Naive Thrombin").

All ingredients were prepared in PBS. The reaction was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of EDTA (25 mM), and centrifugation in a microfuge centrifuge at maximum speed for 3 minutes. The supernatant was discarded and the pellet was washed twice in 1 ml PBS, 0.1% BSA, 5 mM EDTA, 1 mM

PMSF. The radioactivity in the pellet was monitored by a gamma counter.

When competition with non-radioactive polypeptide was carried out, the competing polypeptide was added together with the ^{125}I -labeled polypeptide at the concentrations indicated in the figure legends.

2. Binding of ^{125}I -r31 kD FBD to preformed fibrin clots (Reaction II):

The reaction mixture contains the same components as indicated for Reaction I, except for the ^{125}I -r31 kD. The first incubation was carried out at 37°C for 30 minutes, and only then the ^{125}I -r31 kD was added and the reaction was further incubated for a second period of 30 minutes. Reaction II was terminated and measured as described above for Reaction I.

Results

A. Binding of ^{125}I -r31 kD to fibrin: effect of thrombin and Ca^{++}

The effect of thrombin and Ca^{++} on ^{125}I -r31 kD binding to fibrin during clot formation (Reaction I) or to preformed clots (Reaction II) was studied in citrated human whole blood (Figure 17).

Hirudin, a specific inhibitor of thrombin (25) reduced the binding of ^{125}I -r31 kD to the fibrin clot in Reaction I, indicating that thrombin is needed for the binding. When thrombin is inhibited there is a reduction in clot formation and less fibrin is available for binding. The addition of citrate to blood significantly reduces the

concentration of free Ca^{++} in the serum and therefore the addition of Ca^{++} for fibrin clot formation is obligatory. However, binding of r31 kD to a preformed clot is reduced when carried out in serum which has been already depleted of free Ca^{++} ions by the preformed clot. Addition of Ca^{++} to the Ca^{++} depleted serum increases the r31 kD binding. This effect of Ca^{++} on binding was dramatically demonstrated when binding was measured in PBS. The addition of Ca^{++} increased the binding, even to a higher extent than observed in reaction I. Thus, both clot formation and r31 kD binding are Ca^{++} ion dependent.

Thus, binding of ^{125}I -r31 kD to fibrin in Reaction II increases when the assay is carried out in PBS (Phosphate Buffer Saline solution) instead of serum.

B. Release of ^{125}I -r31 kD from fibrin clot by Plasmin

In order to determine whether the ^{125}I -r31 kD is covalently bound to the fibrin in the clot, plasmin, which is known (26,27) to cleave the N-terminal domain of plasma derived FN between amino acids Arg^{259} and Thr^{260} was added to the clot after ^{125}I -r31 kD was bound. The incubation with plasmin was carried out for various time intervals (Figure 18).

The results demonstrate that plasmin caused a reduction in the radioactivity bound to the clot (pellet). Reduction of radioactivity in the pellet was time dependent and could be attributed to the fact that plasmin cleaved the r31 kD FBD. The amount of radioactivity monitored in the supernatant increased with time. Upon loading of the plasmin soluble reaction fraction (the supernatant) on SDS polyacrylamide gels it is possible to detect the shortened form of the cleaved ^{125}I -

r31 kD. The release of ^{125}I -r31 kD from the fibrin clot occurs only by plasmin cleavage and not by heating with a solution containing SDS, EDTA and β -mercaptoethanol to 100°C, thus demonstrating that ^{125}I -r31 kD is covalently bound to the fibrin clot.

3. Binding of ^{125}I -r31 kD to fibrin during clot formation: effect of unlabeled r31 kD and other molecules

The ability of various cold recombinant and plasma derived FBD preparations as well as human plasma derived fibronectins at different concentrations to interfere with the binding of ^{125}I -rFBD to fibrin during clot formation (Reaction I) was studied (Figure 19). The results obtained demonstrate that the binding of 0.15 μM ^{125}I -rFBD to fibrin was similar or even increased in the presence of unlabeled r31 kD, unlabeled plasma derived 31 kD or unlabeled FN at concentrations up to 20 folds excess (3 μM).

However, when the amount of newly formed fibrin clot was reduced, using either suboptimal concentrations of CaCl_2 and thrombin (1 mM and 0.3 units/ml, respectively), or by reducing the volume of the blood in the reaction to 1/10 of the amount originally specified, the competition between the unlabeled FBD and the binding of ^{125}I -FBD for binding to the clot became significant.

In order to further study the specificity of the FBD binding reaction, we compared the binding of r31 kD to the binding of its reduced forms (Example 5).

For these studies we used one batch of reduced-carboxamidated plasma derived 31 kD, one batch of reduced-

carboxamidated r31 kD and one batch of fully-reduced r31 kD all prepared as described in Example 5. Surprisingly, these various reduced forms of 31 kD FBD caused a dramatic reduction in the binding of the ^{125}I -FBD (reduction of 40-80% by 0.3-3.0 μM ; Figure 19). We have also noticed a dramatic decrease in the size of the newly formed clot (Reaction I) in the presence of the fully reduced FBD. Clot formation was totally inhibited in the presence of high concentrations of the fully reduced FBD (above 5 μM).

Since a similar inhibitory effect was exhibited by the various reduced forms of the 31 kD proteins on the binding of refolded ^{125}I -FBD to a preformed fibrin clot (Reaction II), the possibility of interference with the crosslinking reaction of the FBD to the fibrin clot catalyzed by the serum transglutaminase factor XIIIa, was suggested.

4. Binding of ^{125}I -FBD to the fibrin thrombi (Reaction II): effect of transglutaminase inhibitors

Transglutaminases are a class of calcium ion-dependent enzymes that catalyze an amidation reaction in which a carboxamide group of peptide bound glutaminy residues and primary amines, including the epsilon amino group of peptide bound lysyl residues, are crosslinked. Plasma FN is a substrate for transglutaminase from plasma, factor XIIIa, (thrombin activated blood coagulation factor XIII) or liver; FN can be crosslinked to itself, fibrin and collagen.

The glutaminy residues which are susceptible to factor XIII crosslinking are localized in the FBD region of FN (28).

The binding of ^{125}I -FBD to preformed fibrin clot (Reaction II) in the presence of various concentrations of the primary amines spermidine and putrescine, the classical transglutaminase inhibitors, was studied (Figure 20). The reaction was 50% inhibited by about 5 mM spermidine or putrescine. In parallel to the expected inhibition by the primary amines, a dramatic inhibition of the binding was also observed by the reduced-carboxamidated FBD, with a half maximal reduction at around 2.5 μM .

5. Binding of ^{125}I -r31 kD to preformed fibrin clot: effect of aging on binding

For imaging purposes, it was important to determine the effect of clot aging on FBD binding capacity.

The binding of ^{125}I -r31 kD to preformed fibrin clot was determined. ^{125}I -r31 kD was added to the preformed clots (1, 4 or 24 hours) and binding to fibrin was monitored (Figure 21).

The binding of ^{125}I -r31 kD to the fibrin clot was higher after 24 hours than the binding after 1 or 4 hours, presumably due to the fact that the clot formed became larger with time.

As indicated in experiment I of this example, the presence of free Ca^{++} ions in citrated blood is important in order to obtain optimal binding and crosslinking of ^{125}I -r31 kD to the fibrin clot. Thus, in serum where Ca^{++} was already depleted by the preformed clot the binding of ^{125}I -r31 kD to the fibrin clot was low while in the PBS containing 5 mM CaCl_2 , the binding was high at all incubation times.

When 300 µg/ml of plasma derived FN was added as competitor in the presence of Ca⁺⁺, there was a 20% reduction in ¹²⁵I-r31 kD binding to the fibrin clot probably due to some competition on the available fibrin binding sites.

5
6. Binding of ¹²⁵I-r31 kD to "Naive" thrombi (Reactions I and II): effect of thrombi age on the binding

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10 The ability to differentiate between "old" (preformed) and "newly formed" thrombi, is an important requirement of a probe for thrombus imaging. Figure 22 shows experiments designed to compare ¹²⁵I-r31 kD binding to "old" and "newly formed" clots. In the first experiment
15 ¹²⁵I-r31 kD was added at the same time as the initiation of clot formation ("newly formed") and was allowed to interact with the clot during seven days of incubation. It is evident that FBD is incorporated efficiently and the amount incorporated increased during the first two
20 days, probably representing an increase in the clot size during that period. In the second experiment ¹²⁵I-r31 kD was added to aged clots (1-7 days old) for a limited period of 2 hours ("preformed"). The extent of FBD incorporation remained constant, regardless of the clot age. Note, however, that binding in this protocol is
25 lower than in that of the first experiment. These experiments suggest that the probe ¹²⁵I-r31 kD may be used in two different protocols to differentiate between "old" clots - thrombi and those which are still in the process of growing.

30
7. Plasma "Thrombin Time" ("TT")

35 The effect of the r31 kD polypeptide on clotting of whole blood was measured using the clinical laboratory parameter defined as Thrombin-Time. In this reaction aliquots

of 100 μ l citrated healthy human plasma were mixed with 100 μ l PBS and either the transglutaminase inhibitor spermidine, or with reduced-carboxyamidated p31 kD or reoxidized-refolded r31 kD. 200 μ l thrombin solution was added to each aliquot while continuously mixing. The time from the addition of the thrombin to the formation of clot was measured, and is expressed as "TT" in seconds (Table B).

8. Binding of 125 I-r31 kD to fibrin (Reaction I): effect of exogenous transglutaminase and "reduced-carboxamidated" FBD

In Section 4 of this example we demonstrated that transglutaminase inhibitors reduce the binding of 125 I-r31 kD to clots. In this section we studied the effects of exogenous transglutaminase from pig liver on the binding reaction.

The results presented in Figure 23 demonstrate that the addition of the exogenous transglutaminase dramatically increases the binding of 125 I-r31 kD to clots, indicating that this enzyme may be a rate limiting step in this reaction.

Moreover, both endogenous and exogenous transglutaminase dependent binding activities were equally decreased by the reduced-carboxamidated FBD (approximately 56% and 72% inhibition by 0.3 μ M and 1.5 μ M "reduced" FBD, respectively).

These results strongly indicate that the "reduced" forms of FBD inhibit the binding of the refolded form of FBD to the fibrin clot by interfering with the transglutami-

nation and cross-linking reaction. This probably causes destabilization of the fibrin clot.

9. Binding of ^{125}I -FBD to Extra Cellular Matrix (ECM)

Adhesive molecules such as von Willebrand factor, fibronectin, fibrinogen, thrombospondin, collagen and laminin bind to ECM formed by removal of endothelial cells. r31 kD FBD can serve for imaging the initial steps in plaque formation at the site of injury. The binding of ^{125}I -r31 kD at various concentrations to ECM was studied in the presence or absence of thrombin. The results demonstrated that the binding of ^{125}I -r31 kD at low concentrations to ECM was not affected by thrombin (0.3 μM). At higher concentrations of the thrombin binding of r31 kD was slightly higher indicating that the number of binding sites naturally present are limited and thrombin digestion might expose additional binding sites (Figure 24).

The fact that ^{125}I -r31 kD binds to ECM indicates that it will be useful for imaging the initial plaque formation in the denudated blood vessel.

5

Table B

THROMBIN TIME ("TT")

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Effect of FBD ("reduced" and "folded") and Related Molecules

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	Thrombin Conc. (μ /ml)	Additions	"TT" Values		
			(Seconds)	(%)	(Δ)
I	2.5	-	25.5	100	-
		1mM Spermidin	44	172	72
		1mM "R" FBD	34	133	33
		2.5 μ M "R" FBD	39	152	52
		2.5 μ M "F" FBD	26.5	103	03
II	1.25	-	55.4	100	-
		1mM Spermidine	114	205	105
		2.5 μ M "R" FBD	81	146	46
		2.5 μ M "F" FBD	58	104	04

"R" FBD = Reduced-carboxamidated p31kD.

"F" FBD = Refolded r31kD.

II. Bacterial Binding Activity

5 The involvement of fibronectin in adhesion to, and invasion of, wounds by a wide range of gram-positive bacteria is well established (18). The fibrin binding domain of authentic plasma derived FN has been shown to interact with high affinity to specific receptors on the surface of bacteria. The sites at which Staphylococcus aureus typically initiates infection are rich in FN, e.g. 10 blood clots and subendothelium. Furthermore, exogenous FN enhances bacterial adhesion to these sites. FN binds to S. aureus through saturable, specific surface protein receptors. Scatchard analysis has revealed high affinity receptors with binding constant of 5×10^{-9} M, and a range 15 of 100-20,000 receptors per bacterium (19). The expression of FN receptors correlates with invasiveness and pathogenicity of the clinical isolates. Removal of the receptors from S. aureus by mechanical means, or by growth of the bacteria in the presence of antibiotics 20 decreases their ability to adhere to FN. As FN is a divalent molecule consisting of multiple functional domains with cell binding and collagen binding activities in addition to bacterial binding, it can anchor the bacteria to the wound via the various components of the 25 extracellular matrix as well as via the FN receptor in tissue cells.

Materials and Methods

Binding of Bacteria to labeled FN or FBD in solution

5 A. Direct binding reaction

Various concentrations of ^{125}I -r31 kD FBD or ^{125}I -FN, were added to 5×10^8 S. aureus bacteria in a PBS solution additionally containing 0.1% Tween and 1% BSA. The final
10 volume was 1 ml. Total radioactivity in the reaction was assayed using a 20 μl aliquot taken immediately after the addition of the bacteria.

The mixture was incubated for 2 hours at 20°C.

15 The amount of binding was assayed by removing 100 μl of the incubation mixture and layering on top of 0.5 ml PBS layered on 3 ml 10% percol, 0.15 M NaCl in a 5 ml siliconized tube. This was then centrifuged at 1,350 x
20 g (4,000 rpm in SW bucket rotor) for 15 minutes at 20°C. The supernatant was aspirated and the pellet assayed for radioactivity.

25 B. Competition with unlabeled FN, FBD and related molecules

The procedure followed was identical to the above procedure except that 3 $\mu\text{g/ml}$ ^{125}I -p31 kD was used and the specified amounts of the competing molecule (FN or FBD)
30 was also added to the initial binding mixture.

II. Binding of radioactively labeled bacteria to im-
obilized FN

5 Plastic vials were coated with 0.3 ml of 50 µg/ml FN, or
1% BSA.

The tubes were incubated with shaking at 4°C overnight.
The tubes were then washed with 5 ml PBS three times.
10 Then 0.3 ml of 1% BSA in PBS was added and the tubes were
further incubated with shaking for 2-3 hours at 20°C (for
blocking free sites).

In indirect-binding experiments, the bacteria were pre-
15 incubated with inhibitor, at 4°C for 2 hours.

The bacteria (4×10^6 pfu/ml, 3 pfu/cpm) were added to
the vials at concentrations indicated in the figure
legends. The final volume of the assay mix was 0.3 ml
20 PBS. The mix was slowly agitated at 4°C for 90-120
minutes.

The tubes were then decanted and washed with 5 ml PBS
three times.

25 5 ml of scintillation-liquid was added when assaying for
binding of ³H-labeled bacteria.

III. Binding of labeled bacteria to catheters

30 Catheters ["UNO" sterile bronchial plastic catheters (18
CH size; Unoplast A/S, Denmark)], were cut to 1 cm and 2
cm pieces, weighed and then cut once lengthwise.

The catheter pieces were incubated with 50 µg/ml FN at 4°C overnight with shaking. The controls were incubated with PBS under the same conditions.

5 The FN solution was then decanted and the catheters were washed three times in PBS. BSA-blocking was performed by adding 1% BSA in PBS for 1-2 hours at 20°C. The catheters were again washed three times with PBS.

10 Bacterial binding was performed as described in the previous section except that the catheter pieces were added to the assay mixture. The final volume used was 3 ml. The reaction was performed in 10 ml tubes. The reaction was incubated for 2 hours at 20°C. For the
15 binding assay ¹²⁵I-S. aureus (4 x 10⁶ pfu/ml, 3 pfu/cpm) were used. (When H3-leucine-labeled S. aureus was used the specific activity was 1 cpm/3.3 x 10³ pfu. Labeling was performed according to P.B. Russel et al. (29). When ¹²⁵I-labeled S. aureus was used the specific activity was
20 1 cpm/3 pfu. Labeling was performed according to A.E. Bolton and W.M. Hunter (30).) The competing molecules at the designated concentrations were preincubated with the bacteria for 30 minutes at 20°C. The catheters were then washed three times with PBS and then directly counted in
25 a gamma counter.

Results

A. Binding of bacteria to ¹²⁵I-FN or FBD in solution

I. Direct Binding

30 Experiments were performed in order to determine the binding of ¹²⁵I-FN or ¹²⁵I-rFBD to S. aureus bacteria in
35 suspension. Various amounts of radioactive FN or r31 kD

were added to 5×10^8 bacteria incubated for 2 hours and then centrifuged over a 10% Percoll-saline solution. Radioactivity was monitored in the pellet (Figure 25).

5 The results showed increased binding of ^{125}I -rFBD (r31 kD) to the bacteria in suspension as compared to the binding of the ^{125}I -FN.

10 This increased binding of ^{125}I -rFBD to S. aureus as compared to ^{125}I -FN binding to S. aureus can be attributed to a higher affinity of a monovalent domain in comparison to bivalent multidomain of intact plasma derived FN.

15 II. Binding of ^{125}I -plasma derived 31 kD FBD (p31 kD) to S. aureus: competition with "native" unlabeled FN. FBD and related molecules

20 A fixed amount of ^{125}I -p31 kD ($3 \mu\text{g/ml}$) was incubated with 5×10^8 bacteria in the presence of increasing amounts of various FBD molecules as competitors (Figure 26).

25 The results demonstrate that "native" FN, p31 kD or rFBD inhibited the binding of ^{125}I -p31 kD to S. aureus in a similar fashion, indicating that rFBD is as active as the natural plasma derived molecules. However, the reduced forms of recombinant or plasma derived FBD only minimally inhibit the binding of ^{125}I -FBD to the bacteria, indicating that proper folding is necessary for binding. A related recombinant polypeptide (33 kD cell binding domain of FN) which does not have a bacterial binding site did not inhibit ^{125}I -pFBD binding to S. aureus.

30

B. Binding of labeled *S. aureus* to immobilized FN

To estimate the capacity of rFBD (r31 kD) to interfere with the adherence of bacteria to the extracellular matrix in wounds, a competition assay was developed. In this assay, adherence of *S. aureus* to plastic surface coated with FN, and the interference of FBD with the binding was measured (see Figure 27).

The results demonstrate that the adhesion of *S. aureus* to FN coated plastic vials was inhibited following pre-incubation of *S. aureus* with FN, pFBD or rFBD. The extent of inhibition by these molecules was similar. A non-related protein, BSA, which does not have *S. aureus* binding sites, did not cause any inhibition in adhesion of radioactive labeled *S. aureus* to FN coated plastic vials.

C. Binding of *S. aureus* to bronchial catheters: effect of FBD and heparin

Catheter sepsis due to various species of *S. aureus* contributes to the high incidences of serious clinical complications.

We have examined the ability of *S. aureus* to bind FN coated catheters. Figure 28 demonstrates that the binding of *S. aureus* to the FN coated catheters is quite high, approximately 10^4 PFU/cm².

Preincubation of bacteria with increasing concentrations of r31 kD reduced the binding of the bacteria to the catheters. The IC₅₀ for this inhibition is between 0.08

- 0.8 μ M (Figure 28). Similar inhibition was also obtained with r20 kD FBD and p31 kD FBD.

5 Systemic administration of heparin and/or the use of heparin bonded polyurethane catheters is reported to decrease the incidence of thrombosis.

10 Therefore we also measured the inhibitory effect of FBD on S. aureus attachment to the catheters in the presence of 5 μ M heparin. The results (Figure 28) demonstrate that heparin did not affect the binding of the bacteria to catheters, however, r-FBD inhibition of bacterial binding remains constant even in the presence of heparin. This indicates the utility of r31 kD for inhibition of S.
15 aureus colonization and sepsis in a clinical setting, even in the presence of heparin.

Conclusion

20 These results demonstrate that the r31 kD FBD or the plasma derived 31 kD FBD may be used therapeutically in preventing bacterial colonization of wounds. The various FBD polypeptides will be formulated in suitable pharmaceutical formulations well-known to the average man
25 of the art, and then used to "irrigate" or "flood" or treat the wound area for a suitable period of time, thereby preventing bacterial colonization of the wound.

EXAMPLE 8

Adhesion of plasma derived 31 kD FBD to thrombi in a rabbit aorta lesion model

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In order to demonstrate the ability of the fibrin binding domain to adhere to thrombi in vivo, a model of thrombus formation in rabbits was used. In this model, the endothelial layer of a segment of the aortal wall is removed with a balloon catheter, thus exposing the lamina intima. This results in the subsequent formation of a film of thrombi in the lesion area. It has been demonstrated by Uehara et al. (1) that labeled FN given systemically to rabbits exhibited extensive binding to such thrombi, as indicated by a higher specific radioactivity in the lesion part of the aorta as compared to adjacent untreated segments.

20

25

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In order to compare the plasma derived 31 kD FBD polypeptide with intact plasma-derived FN, the two molecules were iodinated with ^{125}I using the ICL method. Two hours after de-endothelialization, the radio-labeled molecules were injected intravenously into rabbits (20 μCi ; 100 $\mu\text{g/kg}$; 3 rabbits per group). At 72 hours after injection, the aortas were removed, the scraped (abdominal) and intact (thoracic) areas were separated and each part was cut into several segments (5-6 segments for the lesion part and 2 segments for the control part). The tissue pieces were weighed and radioactivity counted.

35

Figure 29 summarizes the specific activity values found in the sequential aorta slices in rabbits injected with ^{125}I -FN (rabbits No. 1-3) or ^{125}I -p31 kD FBD (rabbits 4-6). As can be seen in Figure 29, enhanced localization of the labeled molecules was found in the de-endothelialized

segments (thrombus zone). Also more radioactivity was localized in the clots when labeled p31 kD FBD was used than when labeled FN was used.

- 5 These results demonstrate that molecules comprising the FBD moiety should be useful for the imaging of arterial thrombus clots.

- 10 When used for imaging it may be desirable to attach to the FBD (plasma derived or recombinant) other markers useful in imaging such as enzymes or radio-opaque inert molecules.

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EXAMPLE 9

Expression and Fermentation of Additional Fibrin Binding Domain (FBD) Polypeptides

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In Example 2 the expression of a partial r20 kD FBD and the full-length r31 kD FBD was described and in Example 24 an improved procedure for refolding and purification of the 31 kD FBD was disclosed. The construction of plasmids for expression of additional FBD polypeptides is now described.

10

A. Expression of r12 kD FBD polypeptide

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Plasmid pFN 975-25 (Figure 10) expresses the full-length r31 kD FBD of fibronectin and from it plasmid pFN 196-2 which expresses a partial FBD was constructed as shown in Figure 36. This plasmid was transformed into Escherichia coli strain A1645 and thence into Escherichia coli strain A4255 and deposited in A4255 in the ATCC under Accession No. _____. These transformed cells were found to be good expressors of the partial FBD polypeptide in amounts comprising about 5% of the total cellular protein. The polypeptide has a mobility of about 12 kD on reduced SDS polyacrylamide gels as determined from the mobility of the size markers. The polypeptide comprises the first 109 amino acids of fibronectin; it is not yet known if an additional methionine residue is present at the N-terminus of the final polypeptide. Throughout this specification this polypeptide is referred to as the r12 kD polypeptide or the r12 kD FBD.

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B. Expression of a modified 12 kD (12 kD') partial FBD polypeptide

5 Plasmid pFN 975-25 (Figure 10), which expresses the full-length r31 kD FBD, was used to construct plasmid pFN 197-10 which expresses a modified r12 kD polypeptide (r12 kD') as shown in Figure 37. The fibronectin FBD sequence was modified to produce an NdeI site immediately after
10 nucleotide 340. This plasmid was transformed into Escherichia coli strain A1645 and thence into Escherichia coli strain A4255. These transformed cells were found to be good expressors of the modified r12 kD partial FBD in amounts comprising about 5% of the total cellular
15 protein. The polypeptide has a similar mobility to the unmodified 12 kD FBD as determined on reduced SDS polyacrylamide gels. The polypeptide comprises the first 109 amino acids of fibronectin followed by additional amino acids histidine and methionine; it is not yet known
20 if an additional methionine residue is present at the N-terminus of the final polypeptide. This polypeptide is designated the r12 kD' polypeptide or the r12 kD' FBD.

C. Expression of a modified r12 kD FBD fused to the 33 kD cell binding domain

25 Plasmid pFN 197-10 which contains an NdeI site at the 3' terminus of the modified 12 kD FBD was used to construct a plasmid, designated pFN 202-5, which encodes the
30 modified 12 kD FBD fused to the 33 kD cell binding domain (CBD). This construction was performed as shown in Figure 38 where the 33 kD CBD fragment was taken from plasmid pFN 137-2 (deposited in the ATCC under ATCC Accession No. 67910). Plasmid pFN 202-5 was transformed
35 to Escherichia coli strain A1645 and thence to Escherich-

ia coli strain A4255 and is a good expressor (8% of total protein). The 45 kD ppolypeptide consists of the 12 kD FBD fused to the 33 kD CBD (first 109 amino acids of FBD followed by amino acid residues histidine and methionine followed by the CBD commencing with serine; it is not yet known if an additional methionine residue is present at the N-terminus of the final polypeptide).

D. Expression of a 31 kD FBD polypeptide fused to the amino acid sequence DGRGDS

In order to obtain expression of a 31 kD FBD polypeptide fused at the carboxy terminus to the sequence asp-gly-arg-gly-asp-ser (DGRGDS) the following construction was made. Plasmid pFN 975-25 which expresses the 31 kD FBD was digested with PvuII and HindIII and ligated to a synthetic linker as shown in Figure 39. The resulting plasmid, designated pFN 195-4, was used to transform Escherichia coli strain A1645 and thence Escherichia coli strain A4255. These cells were found to be good expressors of the 31 kD-GRGDS polypeptide, at levels of about 8% of total cellular protein. The sequence of this polypeptide is described in the description of Figure 39.

E. Expression of a fused 31 kD FBD-33 kD CBD

In order to obtain expression of a "full length" r31 kD FBD polypeptide fused to the r33 kD CBD the following construction was made.

Plasmid pFN 975-25 which expresses the 31 kD FBD was digested with PvuII and HindIII, and the large fragment resulting was ligated to a synthetic linker and to the r33 kD cell binding domain obtained from plasmid pFN 137-2 after NdeI and HindIII digestion (as shown in Figure

40). The resulting plasmid, designated pFN 194-2, encodes the r31 kD FBD linked to the 33 kD CBD. Plasmid pFN 194-2 was transformed to Escherichia coli strain A1645 and then to Escherichia coli strain A4255, and the resulting cells were low expressors of a 64 kD polypeptide which comprises the 31 kD FBD fused to the 33 kD CBD. The sequence of this polypeptide is described in the description of Figure 40.

Fermentation and growth conditions

The clone expressing the r12 kD FBD polypeptide was fermented in rich medium (yeast extract and casein hydrolysate) containing ampicillin. Growth was carried out at 30°C. Expression was obtained upon induction at 42°C for 2 hours and subsequently bacterial cell cake containing the r12 kD FBD polypeptide was obtained. Similarly, cell cake containing other proteins described above was obtained.

EXAMPLE 10

Refolding and Purification of Recombinant 20 kD and 12 kD Fibrin-Binding Polypeptides of Fibronectin.

5

The process for refolding and purification of the r20 kD and r12 kD polypeptides is made up of three stages:

1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

10

15

1. Crude processing

20

1.1 Washing and extraction of the pellet: The bacterial cell cake, obtained as described in Example 2 for the r20 kD polypeptide and as described in Example 9 for the r12 kD polypeptide, is disrupted and washed essentially as for the r31 kD polypeptide (Example 5); however, changes were introduced in the extraction procedure used for both the r20 kD and the r12 kD polypeptides. The following is an example of the washing and extraction procedure performed on the bacterial cell cake of the r20 kD polypeptide; the r12 kD polypeptide is extracted in a similar way.

25

30

1.2 Procedure: Bacterial cake containing the r20 kD polypeptide was produced as described in Example 2 by fermentation of Escherichia coli strain A4255 harboring plasmid pFN 949-2. A portion of this bacterial cake (14.8 g) was suspended in 10 volumes of 50 mM Tris HCl, 50 mM EDTA (Buffer B), pH 7.5.

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2. Solubilization and refolding of the extracted pellet

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reduced by the addition of 57 μ l of undiluted 8-mercaptoethanol (final concentration: 50 mM) and stirred in the absence of air, i.e., in a sealed container, for 30 minutes. It was then dripped at the rate of about 5 ml/min into 10 volumes (148 ml) of Buffer C, pH 8.0 and allowed to oxidize while being constantly and gently stirred, in an open beaker for 48-72 hours at room temperature. Although at this stage some polypeptide precipitation had already occurred, the suspension, including the precipitate, was dialyzed over 24 hours against 15 volumes of Buffer C, pH 8.5 with three changes of buffer. The dialysate was then subjected to centrifugation for 45 minutes at 15,000 rpm (22,500 x g) in a high-speed Beckman centrifuge equipped with a JA-17 rotor. This removes many contaminant proteins and aggregates of the r20 kD or r12 kD, which have been produced during reoxidation.

3. Purification and Characterization

Since the location of the heparin binding site within the fibrin binding domain was not known, it therefore could not be known in advance if the new shorter r20 kD and r12 kD polypeptides would bind to Heparin-Sepharose. However, we found that the shorter molecules did in fact bind to Heparin-Sepharose.

We found that there was no need for a phenyl-Sepharose column, as in the case of the r31 kD, in order to purify the reoxidized r20 kD or r12 kD polypeptides. In fact, the material could be directly purified on Heparin-Sepharose, but considerable improvement, with respect to removal

of contaminants, incorrectly folded molecules and dimers, was achieved when the sample was chromatographed on a Q-Sepharose column before chromatography on a Heparin-Sepharose column. In some cases, the polypeptide was concentrated on a Pellicon system (Millipore Corp.), using membranes with appropriate cut-off points, i.e., 10 kD for the r20 kD polypeptide and 3 kD for the r12 kD polypeptide, prior to being loaded on the Q-Sepharose column. The Heparin-Sepharose column is also used for concentration of both polypeptides. The following is an example of the purification procedure used in the case of the r20 kD polypeptide.

3.1. Q-Sepharose Chromatography: One-third of the reoxidized r20 kD (47 ml) was applied to a 10 ml column of Q-Sepharose Fast Flow column, which had been pre-equilibrated in Buffer C, pH 8.5 at 1.2 ml/min flow-rate. The flow-through fraction was collected and saved (70 ml). The polypeptides which adhered to the column were eluted with Buffer C, pH 8.5 containing 0.5 M NaCl and the column was regenerated with 0.5 M NaOH.

3.2. Heparin-Sepharose Chromatography: The flow-through from the Q-Sepharose column was applied to a 10 ml column of Heparin-Sepharose pre-equilibrated in pH 8.5 buffer at a flow rate of 0.5 ml/min. The flow-through fraction contained mostly contaminants and incorrectly folded r20 kD polypeptide. The purified (>95% pure) r20 kD polypeptide was eluted in Buffer C, pH 8.5 containing 0.5 M NaCl and the column was regenerated in the same buffer containing additionally 6M Guanidine-HCl.

Representative purification tables for the r20 kD (Table C) and r12 kD (Table D) polypeptides are provided.

5 3.3 Characterization: Supernatants from the processing
of the bacterial cake for both the r20 kD and the
10 r12 kD polypeptide, as well as aliquots from sub-
sequent column fractions, were assayed for
polypeptide and analyzed by SDS-polyacrylamide gel
electrophoresis; their elution profiles were
obtained on a Superose 12 column attached to either
a FPLC or a HPLC. These profiles at various stages
15 of the refolding, as well as of the purification,
are shown for the r20 kD (Figure 52) and the r12 kD
(Figure 53). The purified r20 kD or r12 kD
polypeptides elute as single sharp bands. These
profiles corroborate the results seen on SDS-PAGE
20 gels under non-reducing conditions; the bands of
both the 20 kD and the r12 kD polypeptides samples
are non-diffuse, indicating a single molecular
form. In the case of the r20 kD, the band of the
non-reduced polypeptide runs (as in the case of the
r31 kD polypeptide) faster than that of the reduced
25 form; this is a similar effect to that seen in the
case of the r31 kD polypeptide. However, no such
difference is observed in the case of the r12 kD
polypeptide.

30 These FBD polypeptides are available for
radiolabeling in order to use them as
radiopharmaceuticals for imaging of thrombi and
atherosclerotic lesions. The FBD polypeptides may
also be used for therapeutical inhibition or
prevention of bacterial colonization and sepsis.

5

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35 30 25 20 15 10 5

TABLE C: PURIFICATION OF THE R20 KD FBD

Step	Volume (ml)	Protein Conc (mg/ml)	Total Protein (mg)	Purity ^a (%)	Amount of FBD (mg)	Yield (%)	Degree of purification
Solubilized & Reduced Pellet	14.8	12.4	183.5	35	64.2	100	1
Oxidized Supernatant	148	0.72	106.5	45	48.0	74.7	1.3
Oxidized Supernatant (1/3)	47		33.8		15.2		
Q-Sepharose Flow-through	70	0.20	14.0	80	11.2	54.9	2.3
Heparin- Sepharose 0.5 M NaCl	9	0.71	6.1	98	6.0	29.4	2.8

^a Estimated from either SDS-PAGE gels under reducing conditions or from Sepharose 12 elution profiles.

This is a representative purification table for the refolding and purification of the r20 kd polypeptide, processed as described in Example 10, Sections 2 and 3.

35 30 25 20 15 10 5

TABLE D: PURIFICATION OF THE R12 KD FBD

Step	Volume (ml)	Protein Conc (mg/ml)	Total Protein (mg)	Purity ^a (%)	Amount of FBD (mg)	Yield (%)	Degree of purification
Solubilized & Reduced Pellet	100	6.66	666	10	66.6	100	1
Oxidized ^b Supernatant	500	0.20	100	58	58.0	87.1	5.8
Q-Sepharose Flow-through	500	0.13	65	80	52	78.1	8.0
Heparin- Sepharose 0.5 M NaCl	14	0.75	10.5	98	10.3	15.4	9.8

- Estimated from either SDS-PAGE gels under reducing conditions or from Sepharose 12 elution profiles.
- Concentrated on a Pellicon system.

This is a representative purification table for the refolding and purification of the R12 KD polypeptide, processed as described in Example 10, Sections 2 and 3.

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EXAMPLE 11

Biological Activity of the r31 kD, r20 kD and r12 kD
Fibrin Binding Domain Polypeptides

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The biological activity of the r31 kD FBD was described
(in Example 7) relating to its binding to fibrin clot in
vivo and in vitro, binding to bacteria and binding to
extracellular matrix. In this example, additional
10 results relating to the r31 kD polypeptide are presented
and the biological activity of the 20 kD and 12 kD FBD
polypeptides is demonstrated.

10

In this Example the binding of the recombinant fibrin
15 binding domains to fibrin clots was measured as follows:

15

Binding of ¹²⁵I-rFBD (r31 kD, r20 kD or r12 kD) to a
preformed fibrin clot (two-step Reaction II)

20

Step 1 Formation of fibrin clot: This may be
done in one of two ways:

Either (a) Incubation at 37°C of 20 µl citrated
human whole blood with 5 mM CaCl₂, 1
25 unit/ml thrombin and PBS in a final vol-
ume of 250 µl. The reaction is terminat-
ed after 45 minutes by centrifugation and
washing of the pellet (twice) with 1 ml
PBS;

30

or (b) Incubation at 37°C of 20 µl non-citrated
whole human blood ("naive" blood). The
reaction is terminated after 150 minutes
by centrifugation and washing as in (a).

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Step 2 Binding of the ^{125}I -FBD polypeptide to the
preformed fibrin clot

Clots are incubated at 37°C in a final volume of 250 μl PBS with ^{125}I -rFBD polypeptide. Other constituents may be added as indicated for each experiment. The binding reaction is terminated after 45 minutes by centrifugation and washing three times with PBS. The tubes containing the ^{125}I -rFBD - fibrin pellet were measured for radioactivity in a gamma counter.

Results

A. Metabolic stability of ^{125}I -labeled r31 kD FBD in rats: ex-vivo binding to fibrin versus TCA insolubility

As described in the description of Figure 44, rats were injected intravenously with r31 kD FBD labeled with ^{125}I and blood samples taken at intervals were added to Na citrate. Aliquots of the blood were treated as follows: either (a) treated with 20% TCA and the TCA insoluble counts were measured; or (b) incubated with preformed clot (using 20 μl whole blood from control rat); binding of the ^{125}I -31 kD FBD to preformed clot was measured under the conditions of the two-step Reaction II described above.

The radioactivity was measured by a gamma counter and the activity of each sample was calculated as a percentage of total cpm present in the reaction mixture.

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The results demonstrated in Figure 44 indicate a good correlation between the physical decay of the r31 kD (as measured by the decrease in TCA insolubility) and the functional decay (as measured by the decrease in ex-vivo binding of the r31 kD to a preformed fibrin clot.) However, at the initial stage of the comparative studies there are marked differences; at 30 min. the functional decay is several fold higher than the physical decay. These results, which suggest a much faster decrease of functional stability than physical degradation, can be explained since the main site for the covalent reaction of the FBD with the fibrin clot is the glutamine residue located at the extreme amino terminus of the FBD molecule at amino acid no. 3; this glutamine residue, being located in a 20 amino acid stretch outside the type 1 finger structure, is not protected from degradation by the tertiary structure, which is typical of the rest of the FBD domain.

B. Specificity of binding of r31 kD to fibrin: effect of transglutaminase

The covalent binding of the fibrin binding domain of plasmatic fibronectin to fibrin in a clot is mainly due to the reaction of amino acid no. 3 of fibronectin (glutamine) with fibrin; this binding reaction is enzymatically controlled by the enzyme transglutaminase which specifically recognizes the amino acid sequence containing this glutamine residue.

The following experiment was performed to investigate if transglutaminase is involved in the binding of the recombinant r31 kD FBD to clots. All the exogenous transglutaminase used in the experiments described in

this application is guinea-pig liver transglutaminase (Sigma).

5 The binding of 0.3 μ M solution of the following molecules to preformed fibrin clot derived from 20 μ l of whole human blood was measured in the presence and absence of transglutaminase using the two-step Reaction II described above: 14 C-putrescine-r31 kD FBD, 125 I-r31 kD FBD and 125 I-recombinant bovine growth hormone (control). The 14 C-putrescine-r31 kD protein complex where the glutamine residue at position 3 is blocked by covalent reaction with 14 C-putrescine, was prepared as follows:

10 A solution containing 3 μ M r31 kD FBD, 10 mM CaCl_2 , 0.015 units/ml transglutaminase and 60 μ M 14 C putrescine (specific activity 100 mc/mmole) was incubated at 37°C for 5 hours. The amount of 14 C-putrescine incorporated into the 31 kD FBD was measured by TCA precipitation of an aliquot of this reaction solution and demonstrated the incorporation of an equivalent amount to 2.8-3 μ M solution of 14 C-putrescine into the r31 kD protein; this indicates that more than 90% of the glutamine at position number 3 of the FBD covalently reacted with the 14 C-putrescine. The 14 C-putrescine r31 kD material was stored at 0°C and used within a few days without further treatment. The r31 kD FBD (prepared as described in Example 5) and the control recombinant bovine growth hormone analog (bGH) prepared as described in EPO Publication No. 131843 were labeled with 125 I using the ICl method described in Example 6.

Results

35 The counts bound in the two-step Reaction II in the presence and absence of transglutaminase were obtained

and the ratio of counts bound in the presence and absence of transglutaminase was calculated for each polypeptide tested (see Figure 45). This ratio differs dramatically when intact 31 kD FBD is compared to putrescine-FBD ("blocked" FBD) or to the control bGH. In the two latter cases the ratio of counts is close to 1 which shows that transglutaminase does not affect the binding and total cpm present in the clot is 10-15% of total cpm in the reaction). In the case of the intact 31 kD FBD the ratio is dramatically higher than 1 (in different experiments the ratio varied between 1.8-7 depending on the quality and freshness of the blood and the transglutaminase) and total cpm present in the clot is 40-70% of total cpm in the reaction) i.e., transglutaminase greatly increased the binding of r31 kD polypeptide to the clot.

These results indicate the strong effectiveness of unblocked glutamine at position number 3 for the binding of the r31 FBD polypeptide to the fibrin clot in the presence of transglutaminase.

Other experiments have shown that the addition of transglutaminase to the two-step Reaction II increases the binding of the r20 kD and r12 kD polypeptides to the clot, comparable to the effect observed with the r31 kD.

C. Characterization of r31 kD FBD-fibrin complex by SDS polyacrylamide gel electrophoresis

In order to determine the size of complex formed by the binding of r31 kD to a fibrin clot the following series of experiments (as described in the description of Figure 46) was undertaken. Clots were derived from either 20 μ l whole human blood (A) or 250 μ l of a solution of 0.8 μ M human fibrinogen (B). In some of the fibrinogen experi-

ments dental coils (as described in Example 12) were added to the tubes together with the fibrinogen.

5 The binding of ^{125}I -r31 FBD to the fibrin clot was measured using the two-step Reaction II described above, in the presence of $0.15\ \mu\text{M}$ ^{125}I -r31 kD FBD and $5\ \text{mM}$ CaCl_2 . The reaction was terminated by three times washing with PBS. The pellet, after the various treatments described below, was centrifuged and $15\ \mu\text{l}$ aliquots of the supernatant (i.e., the soluble material) were electrophoresed on polyacrylamide gels which separates the material of molecular weight $>10^6$ from molecular weight $>10^5$ and from lower molecular weight materials. An autoradiogram was produced (Figure 46) which shows the following: in the presence of transglutaminase high molecular weight forms of r31 kD - fibrin complex appears which are resistant to boiling in the presence of the strong ionic detergent SDS and β -mercaptoethanol, which reduces S-S bonds.

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20 Additionally, Figure 46 demonstrates that when 4M urea is included in the boiling reaction the very high molecular weight forms ($>10^6$) are quantitatively converted to the intermediate molecular weight forms ($>100,000$) as expected for hydrophobic bonded aggregates of high molecular weight fibrin clots. The amount of free r31 kD polypeptide in the clots is normally small; this is the material released on boiling with phosphate-saline buffer only. The resistance of the intermediate molecular weight forms to additional treatment with urea supports the involvement of a covalent linkage between the ^{125}I -r31 kD and the fibrin.

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D. Effect of fibronectin and heparin on the binding of
 ^{125}I -r31 kD to preformed fibrin clots

5 (i) Effect of fibronectin (FN)

Human plasma contains substantial levels of FN (300
 $\mu\text{g/ml}$) which potentially could compete with the binding
of ^{125}I -r31 kD polypeptide to preformed clots. Such
10 competition may affect the efficiency of clot
radiolabeling and subsequently the imaging process. To
examine the effect of FN, ^{125}I -r31 kD ($0.15\ \mu\text{M}$) was added
together with purified FN ($1\ \mu\text{M}$) to a preformed clot in
PBS (Figure 47A). Although FN was added in a molar
15 excess of 7 relative to ^{125}I -r31 kD, the binding of the
latter polypeptide was only slightly affected (20%
inhibition). The observation that excess FN does not
compete with ^{125}I -r31 kD binding could be interpreted in
two ways: the number of sites for crosslinking onto the
20 clot is in excess to accommodate both FN and ^{125}I -FBD, or
the affinity of FBD to the clot is much higher than that
of FN. Based on several observations, we believe that
both excess binding sites and higher affinity of the ^{125}I -
r31 kD enable its binding to the clot in the presence of
25 plasma concentrations of FN.

(ii) Effect of heparin

Some radioscintigraphic agents such as ^{111}In -labeled
30 platelets and labeled fibrinogen are ineffective in the
presence of therapeutic heparin. It was important,
therefore, to analyze the effect of heparin on the
incorporation of ^{125}I -r31 kD to the clots. The results
shown in Figure 47B indicate that heparin has no signifi-
35 cant effect on the binding of r31 kD FBD to preformed

clots. Other experiments showed that the same amount of heparin affects dramatically the binding of r31 kD to a fibrin clot during its formation, i.e., Reaction I (see Example 7).

5

E. Comparison of the binding of various recombinant FBD polypeptides and plasmatic FBD to preformed clots

10 To compare the binding to preformed clots of the various recombinant FBD polypeptides (r31 kD, r20 kD and r12 kD) and plasmatic 31 kD FBD, a series of experiments using the two-step Reaction II was carried out as described in Figure 48. The results show that the plasmatic 31 kD
15 binds to a similar level as the r31 kD whereas the r20 kD and r12 kD polypeptides both bind at about half the level of the larger (31 kD) molecule. The level of binding of the r20 kD and r12 kD polypeptides is still sufficiently high to demonstrate the potential of radiolabeled r20 kD
20 and r12 kD polypeptides as radiopharmaceuticals for thrombus imaging. Similar experiments using the r31 kD-DGRGDS polypeptide (Example 9, D and Figure 39) showed that it binds at about the same level as the r31 kD.

25 F. Binding of ^{125}I -r12 kD to fresh or frozen clots

In order to study the effect of freezing the clots prior to use in binding experiments with FBD polypeptides, the following experiment was carried out. Fibrin clots were
30 either used fresh or after storage at -70°C in a two-step Reaction II binding experiment with ^{125}I -r12 FBD, prepared as described in Example 10.

The experiment was carried out as described in the
35 Description of Figure 41 in the presence or absence of

transglutaminase. Figure 49 shows that there is little significant effect of freezing on the abilities of clots to bind r12 kD FBD. Normally, frozen clots without added transglutaminase yield binding results similar to fresh clots in the presence of transglutaminase; there is no effect on the binding reaction when exogenous transglutaminase is added to frozen clots, probably because of the release of endogenous transglutaminase from the frozen red blood cells.

As noted in Section B above, there is a wide range of response to addition of exogenous transglutaminase in Reaction II.

G. Conditions for binding of ^{125}I -r31 kD FBD to pre-formed clots

To investigate the conditions for binding of ^{125}I -r31 kD to preformed clots, the following series of experiments were carried out. The binding of ^{125}I -r31 kD polypeptide to preformed clots formed from citrated or "naive" blood was examined, using the two step Reaction II method, and in the presence or absence of various constituents (calcium, hirudin, transglutaminase). The results are shown in Figure 50. The pattern of results using the "citrated" blood or "naive" blood clots is similar although the binding of the r31 kD polypeptide is higher to citrated blood.

Hirudin (Sigma) is a specific inhibitor of any thrombin-mediated reaction and the hirudin was therefore added in order to investigate the effect of thrombin on the binding reaction (step 2). No effect of hirudin was shown and therefore thrombin has no effect on the binding of the r31 kD polypeptide to the clot. However, the same

amount of hirudin totally inhibits the binding when added at step 1 where fibrin is formed from fibrinogen, as was expected.

5 These results also show that exogenous transglutaminase increases the binding of r31 kD FBD to clots and furthermore that this transglutaminase reaction is dependent on the presence of calcium ions. Since the exogenous transglutaminase used is tissue transglutaminase (in its
10 active form) we expect that the serum transglutaminase, factor XIII, which has to undergo activation by thrombin to form factor XIIIa, will be highly sensitive to hirudin inhibition.

15 H. Conditions for binding ^{125}I -r31 kD FBD to the extracellular matrix (ECM)

The binding of ^{125}I -r31 kD to the extracellular cell matrix of endothelial cells (ECM) was demonstrated in
20 Example 7 (Section 9) and in Figure 24. The binding was now further characterized by examination of the binding of $0.3\ \mu\text{M}$ ^{125}I -r31 kD FBD to ECM in the presence and absence of exogenous transglutaminase; additionally the binding in the presence of transglutaminase was examined
25 in the presence of each of heparin, fibronectin or spermidine.

The results of these experiments are shown in Figure 51 which demonstrates that the binding of the r31 kD FBD to
30 ECM is increased by the addition of transglutaminase. Heparin has no significant effect on the binding whereas spermidine, a known inhibitor of transglutaminase, inhibits the binding. Collagen also inhibits the binding, suggesting the possible involvement of collagen
35 as an acceptor molecule on the matrix of the endothelial

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cells. Fibronectin has little effect on the binding reaction.

- 5 These results give more support to the potential use of radiolabeled recombinant FBD polypeptides as radiopharmaceuticals for imaging the initial plaque formation in denudated blood vessels.

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EXAMPLE 12

Uptake of Recombinant ^{125}I -31 kD FBD and Fragments Thereof
by Stainless Steel Coil-Induced Venous Thrombi in Rats

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The stainless steel coil-induced venous thrombus model in rats was used to study the uptake of labeled r31 kD, r20 kD and r12 kD FBD polypeptides. The model employed was as described by Maffrand et al. [Thrombosis and Haemostasis 59: 225-230 (1988)].

10

Experimental Details

A. Investigation of the uptake of ^{125}I -31 kD by the
stainless steel coil-induced venous thrombus

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Wistar-derived female rats (200-250 g) were anaesthetized by Ketamine HCl plus Xylazin HCl. A midline abdominal incision was made and the inferior vena cava was exposed. A stainless steel wire coil (a dental paste carrier, fine No. 31, 21 mm long) was inserted into the lumen of the vein at the site just below the junction, and the incision was sutured. Each inserted device was individually weighed before insertion and each weight recorded. Three hours after the operation, the animals were given an i.v. injection of 1 ml of 0.9% NaI solution in order to saturate the thyroid iodide pool. One hour later, the rats received an i.v. injection of ^{125}I -r31 kD FBD (5×10^6 cpm; 100 $\mu\text{g/kg}$). The r31 kD polypeptide was labeled as described in Example 6. At 24 hours after the administration of the labeled polypeptide, blood was drawn by cardiac puncture, and the rats were sacrificed. The segment of the vein carrying the coil was removed while taking care to drain away all residual blood. In one group, the segments carrying the coil were weighed as

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such and taken for measurement of radioactivity (the "Thrombus in Situ" group). In another group the vein sections were incised longitudinally, and the coils carrying the thrombi were carefully removed, weighed and the radioactivity was measured. The blood radioactivity levels were measured using peripheral blood.

Calculation of the results:

In the two groups, the initial weight of each coil was subtracted from its final weight, and the specific radioactivity in each case was calculated by division of the cpm value by the net weight. The specific activity of the peripheral blood samples was also calculated.

Results:

At 24 hours, the blood levels of radioactivity were around 5000-10,000 cpm/g, while in the isolated blood clot the specific radioactivity was around 300,000 cpm/g, i.e., 30 to 60 fold higher (see Figure 42). When the entire segment of the vein carrying the clot was included in the analysis, and a so-called "specific radioactivity" value calculated, the resultant values were 4-5 fold higher than those of the blood, thus indicating that a good signal-to-noise ratio may be obtained for gamma-camera imaging of blood clots in vivo using labeled r31 kD FBD.

The effect of heparin pretreatment was studied in this model. This kind of experiment is essential because patients that are candidates for thrombus imaging are usually treated with this anticoagulation agent. In order to study this question, a group of rats were treated with heparin (500 units/rat intravenously) 10

minutes before administration of the labeled polypeptide. This treatment of heparin did not affect the uptake of label, as measured 24 hours later.

5 These results demonstrate that thrombus imaging using the FBD of FN may be done in the presence of heparin.

10 B. Comparison of recombinant 12 kD, 20 kD and 31 kD-FBD polypeptides in the stainless steel coil-induced venous thrombus model

15 The three recombinant polypeptides were labeled with ¹²⁵I as described in Example 6 and utilized in the rat model as described in A above. The results, shown in Figure 43, indicate that each of the three molecules was specifically localized in the clots as compared to the blood, by comparing the specific radioactivities; the specific radioactivity of the clots appeared to be higher with the longer molecules than the shorter polypeptides (143,000, 78,500 and 63,000 cpm/g clot for the r31 kD, r20 kD and r12 kD polypeptides, respectively), but the differences were not statistically significant. The specific radioactivity values for blood (after 24 hours) were similarly related to the molecular size (7040, 5016 and 3300 cpm/g for the r31 kD, r20 kD and r12 kD polypeptides, respectively) and might reflect differences in the blood clearance rates of these molecular species. Hence, the calculations of the ratio of clot to blood specific radioactivity resulted in values that were similar for the three different polypeptides, and ranged around 20. These results suggest that all three FBD species (or other fragments of the FBD) could serve for thrombus imaging.

EXAMPLE 13

Labeling of the fibrin binding domain polypeptides for
imaging atherosclerotic lesions and thrombi

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The fibrin binding domain polypeptides described in this application (the r31 kD, the r20 kD and the r12 kD polypeptides), or other FBD fragments, may be radioactively labeled to carry a radiotracer to a thrombus in order to permit its external detection by gamma camera imaging. This application discloses in Example 6 the labeling of these three polypeptides by means of iodine-125 (^{125}I), which has a long half life of 60 days.

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Another radioiodine is iodine-131 (^{131}I) which may be used to label the FBD polypeptides using known methods such as described by Uehara et al (1). However, ^{131}I has a relatively long half life of 8 days.

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Optimally, a radiopharmaceutical for clinical imaging of atherosclerotic lesions and thrombi should yield positive results within the first few hours after injection (33). For such a test a shorter lived radiolabel could be used. Recent studies have suggested that indium-111 (^{111}In) or technetium-99m ($^{99\text{m}}\text{Tc}$) may be more suitable as radio-tracers, since they have half-life of 67 hours and 6 hours, respectively (32); another short-lived low energy label is iodine-123 (^{123}I) with a half-life of 13.3 hours.

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We have therefore labeled the r31 kD, r20 kD and r12 kD polypeptides and the plasmatic 31 kD fragment by means of ^{111}In using the method described for human serum albumin by Hnatowich, D.J., Layne, W.W. and Childs, R.L. in J. Appl. Radiat. Inst. 33: 327 (1982). Preliminary experi-

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ments have shown that the labeled FBD polypeptides bind to preformed thrombi in vitro, measured by the two-step Reaction II (Example 11) and to thrombi in vivo measured by the model described in Example 12, and giving a high thrombus:blood ratio in the range of 80-200 after 24 hours.

The labeling of the FBD polypeptides by ^{99m}Tc may be carried out using known methods (21, 33, 34, 35). ^{99m}Tc is a very suitable diagnostic single photon radionuclide because of its short half-life, a detection level of 140 KeV with the gamma counter, no particulate radiation and inexpensive, convenient availability. These attributes allow the routine administration of doses of 30m Ci that result in high photon-flux levels facilitating lesion detection by single photon emission computerized tomography (32, 35).

Other radiolabels which may be used to label the FBD polypeptides include krypton-81m (^{81m}Kr) and xenon-133 (^{133}Xe), which has a half-life of 5.3 days, as reviewed by Knight (4). Another potential radiolabel is gallium-67 (^{67}Ga) as described by Yamamoto (36); ^{67}Ga has a half-life of 78 hours.

NMRI, ultrasound and X-ray imaging with metal chelates are described in U.S. Patent 4,647,447. In addition, antibody coupling with metal chelates is mentioned at column 7, line 42. Monoclonal antibodies labeled with polymeric paramagnetic chelates and their use in NMRI methods have also been described [Shreve, P. et al., Magnetic Resonance in Medicine 3: 336-340 (1986) and Brady, T. et al. in Proceedings of the Society of Magnetic Resonance in Medicine, Second Annual Meeting, Soc. of Magnetic Resonance in Medicine, Inc., San

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What is claimed is:

1. An imaging agent which comprises a polypeptide
labeled with an imageable marker, such polypeptide
having an amino acid sequence substantially present
in the fibrin binding domain of naturally-occurring
human fibronectin and being capable of binding to
fibrin.
2. A composition comprising an effective imaging
amount of the imaging agent of claim 1 and a
physiologically acceptable carrier.
3. An agent of claim 1, wherein the marker is a
radioactive isotope, an element which is opaque to
X-rays, or a paramagnetic ion.
4. An agent of claim 3, wherein the marker is a
radioactive isotope.
5. An agent of claim 4, wherein the radioactive
isotope is indium-111.
6. An agent of claim 4, wherein the radioactive
isotope is technetium-99m.
7. An agent of claim 4, wherein the radioactive
isotope is iodine-123, iodine-125, iodine-131,
krypton-81m, xenon-133, or gallium-67.
8. An agent of claim 1, wherein the polypeptide is a
31 kD polypeptide corresponding to an amino acid
sequence present in the fibrin binding domain of

human fibronectin and having the amino acid sequence of amino acids 1-262 as shown in Figure 1.

9. An agent of claim 1, wherein the polypeptide comprises a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1.

10. An agent of claim 9, wherein the polypeptide comprises less than about 20 additional amino acids.

11. An agent of claim 1, wherein the polypeptide is a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

12. A method for imaging a fibrin-containing substance which comprises contacting the fibrin-containing substance to be imaged with the agent of claim 1 under conditions such that the agent binds to the fibrin-containing substance and imaging bound agent and thereby imaging the fibrin-containing substance.

13. A method of claim 12, wherein the fibrin-containing substance is a thrombus.

14. A method of claim 12, wherein the fibrin-containing substance is atherosclerotic plaque.

15. A method for imaging a fibrin-containing substance in a subject which comprises:

(a) administering to the subject a composition of claim 2 under conditions permitting the imaging agent contained therein to enter the blood stream and bind to fibrin present in the blood vessels;

(b) imaging bound agent within the blood vessels; and thereby

(c) imaging the fibrin-containing substance.

16. A method of claim 15, wherein the fibrin-containing substance is a thrombus.

17. A method of claim 15, wherein the fibrin-containing substance is atherosclerotic plaque.

18. A method of claim 12, wherein the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-262 as shown in Figure 1.

19. A method of claim 12, wherein the polypeptide comprises a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1.

20. A method of claim 19, wherein the polypeptide comprises less than about 20 additional amino acids.
- 5 21. A method of claim 12, wherein the polypeptide is a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.
- 10 22. A method of claim 12, wherein the marker is a radioactive isotope, an element which is opaque to X-rays, or a paramagnetic ion.
- 15 23. A method of claim 12, wherein the imaging is carried out using a gamma camera.
- 20 24. A plasmid for expression of a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a
- 25 suitable host cell.
- 30 25. A plasmid of claim 24, wherein the polypeptide is about a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin.
26. A plasmid of claim 24, wherein the polypeptide comprises about a 20 kD polypeptide corresponding

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to an amino acid sequence present in the fibrin binding domain of human fibronectin.

5 27. A plasmid of claim 24, wherein the polypeptide is about a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin.

10 28. A plasmid of claim 25, wherein the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-262 as shown in Figure 1.

15 29. A plasmid of claim 26, wherein the polypeptide comprises a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in
20 Figure 1.

25 30. A plasmid of claim 29, wherein the polypeptide comprises less than about 20 additional amino acids.

30 31. A plasmid of claim 27, wherein the polypeptide is a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

35 32. A plasmid according to claim 28 designated pFN 975-25 and deposited in Escherichia coli strain A4255 (F⁺) under ATCC Accession No. 67832.

33. A plasmid according to claim 30 designated pFN 949-2 and deposited in Escherichia coli strain A1645 under ATCC Accession No. 67831.

5 34. A plasmid according to claim 31 designated pFN 196-2 and deposited in Escherichia coli strain A4255 under ATCC Accession No. ____.

35. A cell which comprises the plasmid of claim 24.

10

36. A bacterial cell according to claim 35.

37. An Escherichia coli cell according to claim 36.

15

38. An Escherichia coli cell according to claim 37, wherein the plasmid is the plasmid designated pFN 975-25 and wherein the cell is deposited under ATCC Accession No. 67832.

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39. An Escherichia coli cell according to claim 37, wherein the plasmid is designated pFN 949-2 and wherein the cell is deposited under ATCC Accession No. 67831.

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40. An Escherichia coli cell according to claim 37, wherein the plasmid is designated pFN 196-2 and wherein the cell is deposited under ATCC Accession No. ____.

30

41. A method of producing a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises treating the cell according to claim 35 so that the DNA directs expression of the

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polypeptide and the cell expresses the polypeptide and recovering from the cell the polypeptide so expressed.

- 5 42. A method of producing a 31 kD polypeptide
corresponding to an amino acid sequence present in
the fibrin binding domain of naturally-occurring
human fibronectin which comprises treating an
10 Escherichia coli cell comprising the plasmid of
claim 28 so that the DNA directs expression of the
polypeptide and the cell expresses the polypeptide
and recovering from the cell the polypeptide so
expressed.
- 15 43. A method of producing a 20 kD polypeptide fragment
corresponding to an amino acid sequence present in
the fibrin binding domain of naturally-occurring
human fibronectin which comprises treating an
20 Escherichia coli cell comprising the plasmid of
claim 29 so that the DNA directs expression of the
polypeptide and the cell expresses the polypeptide
and recovering from the cell the polypeptide so
expressed.
- 25 44. A method of producing a 12 kD polypeptide fragment
corresponding to an amino acid sequence present in
the fibrin binding domain of naturally-occurring
human fibronectin which comprises treating an
30 Escherichia coli cell comprising the plasmid of
claim 31 so that the DNA directs expression of the
polypeptide and the cell expresses the polypeptide
and recovering from the cell the polypeptide so
expressed.

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45. A purified polypeptide substantially free of other substances of human origin which has an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

46. A polypeptide of claim 45, wherein the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-262 as shown in Figure 1.

47. A polypeptide of claim 45, wherein the polypeptide comprises a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1.

48. A polypeptide of claim 47, wherein the polypeptide comprises less than about 20 additional amino acids.

49. A polypeptide of claim 45, wherein the polypeptide is a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

50. A polypeptide of claim 45 fused to a second polypeptide which comprises a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin.

51. A 45 kD fused polypeptide of claim 50, wherein the polypeptide is a 12 kD polypeptide and the second polypeptide is a 33 kD polypeptide.
- 5 52. A polypeptide of claim 45, wherein the amino acid sequence GRGDS is fused to the N-terminus of the 31 kD polypeptide.
- 10 53. A 64 kD fused polypeptide of claim 50, wherein the polypeptide is a 31 kD polypeptide and the second polypeptide is a 33 kD polypeptide.
- 15 54. A plasmid for expression of the 45 kD fused polypeptide of claim 51 designated pFN 202-5.
- 20 55. A plasmid for expression of the polypeptide of claim 52 designated pFN 195-4.
- 25 56. A plasmid for expression of the 64 kD fused polypeptide of claim 53 designated pFN 194-2.
- 30 57. A method of treating a subject susceptible to, or afflicted with, a bacterial infection which comprises administering to the subject an amount of a polypeptide of claim 45 effective to prevent or treat the bacterial infection.
- 35 58. A method of claim 57, wherein the subject is susceptible to, or afflicted with, a bacterial infection due to the presence of a catheter or an implant in the subject.
59. A coated medical device comprising a medical device and the polypeptide of claim 45 applied as a coating to the surface of the medical device.

60. A coated medical device of claim 59, wherein the medical device is a catheter.

5 61. A method of minimizing risk of bacterial infection associated with use of medical devices which comprises:

(a) applying the polypeptide of claim 45 as a coating to a surface of the device; and

10 (b) employing the resulting coated device rather than an uncoated device.

15 62. A method of claim 61, wherein the medical device is a catheter.

20 63. A method of minimizing risk of bacterial infection associated with use of medical devices which comprises employing the coated device of claim 59 rather than an uncoated device.

25 64. A method of refolding and reoxidizing a polypeptide having an amino acid sequence substantially present in fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide.

30 65. A method of claim 64, wherein the thiol-containing compound is selected from the group consisting of glutathione, thioredoxin, β -mercaptoethanol, and cysteine.

66. A method of claim 64, wherein the thiol-containing compound is β -mercaptoethanol and the disulfide is produced in situ by introduction of air.
- 5 67. A method of claim 64, wherein the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.
- 10 68. A method of claim 64, which additionally comprises contacting the polypeptide with a denaturant.
69. A method of claim 68, wherein the denaturant is guanidine hydrochloride or urea.
- 15 70. A method of claim 64, wherein the polypeptide is at a low concentration.
71. A method of claim 70, wherein the concentration is below 600 $\mu\text{g/ml}$.
- 20 72. A method for recovering a purified biologically active polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin from a cell in which the polypeptide has been produced by means of expression of a plasmid containing DNA encoding the polypeptide which comprises:
- 25 (a) disrupting the cell so as to produce a lysate containing the polypeptide;
- 30 (b) centrifuging the lysate so as to concentrate the polypeptide;
- 35

- (c) separating the concentrated polypeptide;
- (d) solubilizing the separated, concentrated polypeptide;
- (e) refolding and reoxidizing the solubilized polypeptide;
- (f) separating the refolded and reoxidized polypeptide; and
- (g) recovering the purified, refolded and reoxidized polypeptide.

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73. A method of claim 72, wherein the refolding and reoxidizing comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide.

20

74. A method of claim 73, wherein the thiol-containing compound is selected from the group consisting of glutathione, thioredoxin, β -mercaptoethanol, and cysteine.

25

75. A method of claim 73, wherein the thiol-containing compound is β -mercaptoethanol and the disulfide is produced in situ by introduction of air.

30

76. A method of claim 73, wherein the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.

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77. A method of claim 73, which additionally comprises contacting the polypeptide with a denaturant.

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78. A method of claim 77, wherein the denaturant is guanidine hydrochloride or urea.
79. A method of claim 73, wherein the polypeptide is at a low concentration.
80. A method of claim 79, wherein the concentration is below 600 $\mu\text{g/ml}$.
81. A method of claim 72, wherein the separating of the concentrated polypeptide in step (c) comprises chromatography.
82. A method of claim 81, wherein the chromatography comprises Heparin-Sepharose chromatography.
83. A method of inhibiting thrombus formation in a subject susceptible to thrombus formation which comprises administering to the subject an amount of a polypeptide of claim 45 effective to inhibit thrombus formation.
84. A method of inhibiting thrombus formation in a subject susceptible to thrombus formation which comprises administering to the subject an amount of a polypeptide of claim 50 effective to inhibit thrombus formation.
85. A polypeptide in accordance with claim 45 bound to a thrombolytic agent.
86. A polypeptide bound to a thrombolytic agent in accordance with claim 85, wherein the thrombolytic agent is selected from the group consisting of: tissue plasminogen activator (TPA), urokinase,

streptokinase, prourokinase, Anisoylated
Plasminogen-Streptokinase Activator Complex
(Eminase™), TPA analogs, or a protease.

- 5 87. A method for achieving thrombolysis of a thrombus
which comprises administering to a subject an
amount of the polypeptide of claim 85 effective to
achieve thrombolysis.

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FIBRIN BINDING DOMAIN POLYPEPTIDES
AND USES AND METHODS OF PRODUCING SAME

5

ABSTRACT OF THE DISCLOSURE

10 This invention provides an imaging agent which comprises
a polypeptide labeled with an imageable marker, such
polypeptide having an amino acid sequence substantially
present in the fibrin binding domain of naturally-
occurring human fibronectin and being capable of binding
to fibrin. The invention further provides a method
15 wherein the imaging agent is used for imaging a fibrin-
containing substance, i.e., a thrombus or atherosclerotic
plaque. Further provided are plasmids for expression of
polypeptides having an amino acid sequence substantially
present in the fibrin binding domain of naturally-
20 occurring human fibronectin and being capable of binding
to fibrin, hosts containing these plasmids, methods of
producing the polypeptides, methods of treatment using
the polypeptides, and methods of recovering, refolding
and reoxidizing the polypeptides. The invention also
25 provides for purified polypeptides substantially free of
other substances of human origin which have an amino acid
sequence substantially present in the fibrin binding
domain of naturally-occurring human fibronectin and which
are capable of binding to fibrin.

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Figure 1A

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[illegible]

Figure 1C

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Figure 14'

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4810	4820	4830	4840	4850	4860	4870	4880	4890	4900	4910	4920	4930	4940	4950	4960	4970	4980	4990	5000	5010	5020	5030	5040	5050	5060	5070	5080	5090	5100	5110	5120	5130	5140	5150	5160			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36			
3790	3800	3810	3820	3830	3840	3850	3860	3870	3880	3890	3900	3910	3920	3930	3940	3950	3960	3970	3980	3990	4000	4010	4020	4030	4040	4050	4060	4070	4080	4090	4100	4110	4120	4130	4140			
4150	4160	4170	4180	4190	4200	4210	4220	4230	4240	4250	4260	4270	4280	4290	4300	4310	4320	4330	4340	4350	4360	4370	4380	4390	4400	4410	4420	4430	4440	4450	4460	4470	4480	4490	4500			
4510	4520	4530	4540	4550	4560	4570	4580	4590	4600	4610	4620	4630	4640	4650	4660	4670	4680	4690	4700	4710	4720	4730	4740	4750	4760	4770	4780	4790	4800	4810	4820	4830	4840	4850	4860			
4870	4880	4890	4900	4910	4920	4930	4940	4950	4960	4970	4980	4990	5000	5010	5020	5030	5040	5050	5060	5070	5080	5090	5100	5110	5120	5130	5140	5150	5160	5170	5180	5190	5200	5210	5220			
5230	5240	5250	5260	5270	5280	5290	5300	5310	5320	5330	5340	5350	5360	5370	5380	5390	5400	5410	5420	5430	5440	5450	5460	5470	5480	5490	5500	5510	5520	5530	5540	5550	5560	5570	5580			
5590	5600	5610	5620	5630	5640	5650	5660	5670	5680	5690	5700	5710	5720	5730	5740	5750	5760	5770	5780	5790	5800	5810	5820	5830	5840	5850	5860	5870	5880	5890	5900	5910	5920	5930	5940			
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6310	6320	6330	6340	6350	6360	6370	6380	6390	6400	6410	6420	6430	6440	6450	6460	6470	6480	6490	6500	6510	6520	6530	6540	6550	6560	6570	6580	6590	6600	6610	6620	6630	6640	6650	6660			
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Figure 16

[illegible]

Figure 1H

[illegible]

Pair 1

0 2 3'- GTATACGTCGCTGCTTACCAAGTCGGGGTCAGGGGCAACCGACAGTCAGTTTCGTTCCGGCCAAACAACTAA - 5

Page 2

CTGTACCTTTGTGATAGTCTATTAGTGTCAACCCTCGCTGGATGATCCATT - 5'

Pair 3

ACACACCAACATGACCAATACCTCCTCGGTCCAAAATGACGCTTCAATTGACCTCGACTTCT - 5

Pair A

3'-
GACGAACTGTTCAATGACCCCTGTGATGGCTCACCACCTGTGATACTCCAG-5'

Deborah **W. Poff** is an Associate Professor of Management Science at the University of California, Berkeley, and a Senior Lecturer at the University of California, San Diego. She is also a Senior Lecturer at the University of California, Berkeley, and a Senior Lecturer at the University of California, San Diego. She is also a Senior Lecturer at the University of California, Berkeley, and a Senior Lecturer at the University of California, San Diego. She is also a Senior Lecturer at the University of California, Berkeley, and a Senior Lecturer at the University of California, San Diego.

Figure 2B

Pair 5

0 9 5'- GACTCCATGATCTGGAGCTGTACCTGCATCGGGCTGGGCGAGGAGATAAGCTGACC - 3'
010 GATTCTGAGGTACTAGACCTGACATGACGTAGCCCCGACCCGCTCCCTTATTC - 5'

Pair 6

011 5'- ATGCCAACGCTGCCATGAGGGGGTCAGTCCCTACCAATGGTGACACCTGGAGGAGACCACATGAGACT - 3'
012 GACATGCTAGCGTTGCGACGGTACTTCCCCCAGTCAGGATGGTCTMCCACTGTGACCTCCTCTGGTGTACTGTGACCACCA - 5'

Pair 7

013 5'- GGTGGTACATGTGAGGTGTGTGTCTTGTAATGMAAMGGAGATGGACCTGCAGGCCCATAGCTGAG - 3'
014 3'- TGTACATCTCACAACACACAGAACCATTAACCTTTCCTCTTACCTGGACGTTGGGTATCGACTCCTAG - 5'

11/62

Figure 3

Synthesis of F.N. Gene (14 - 472 b.p.)

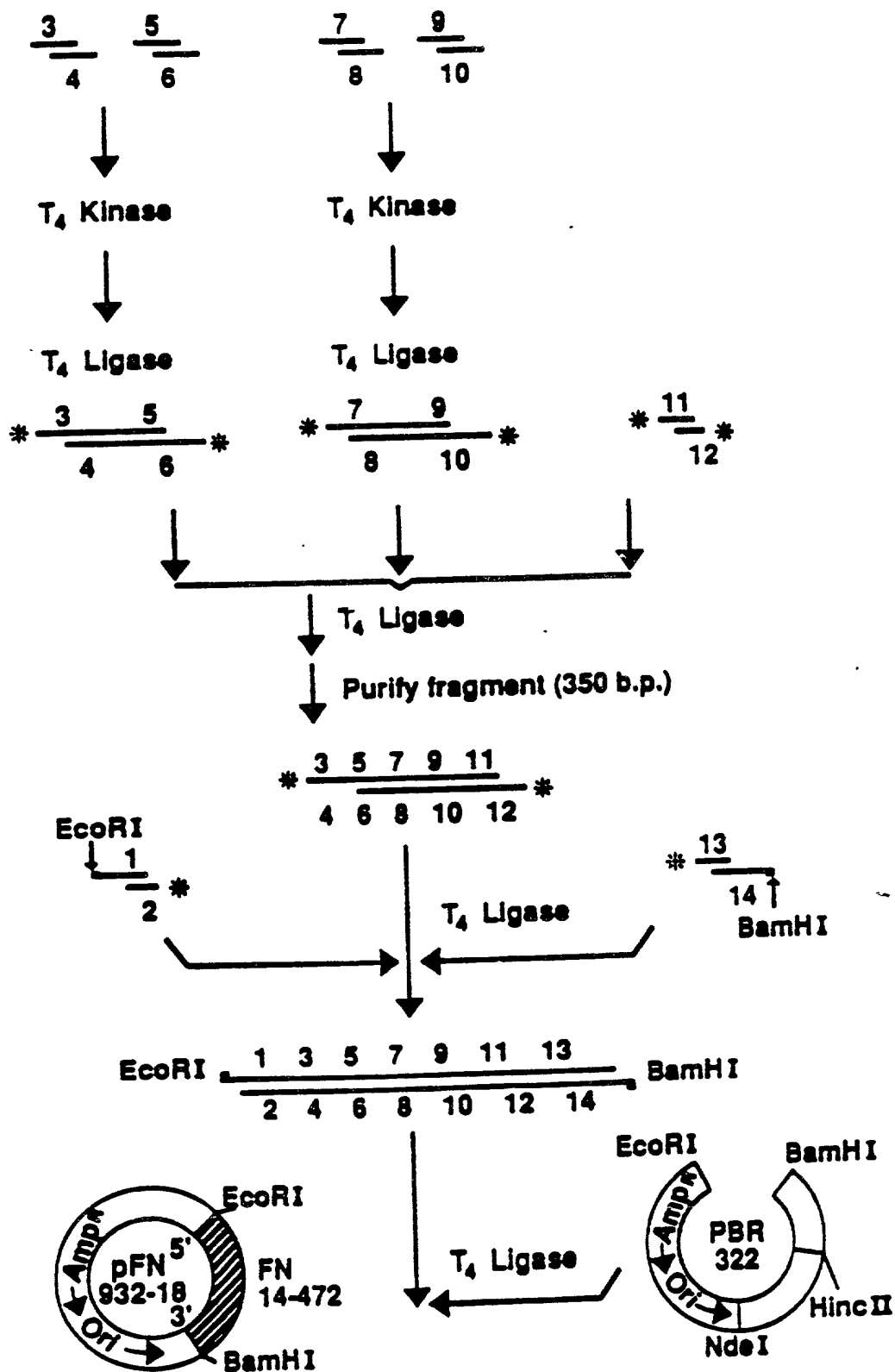
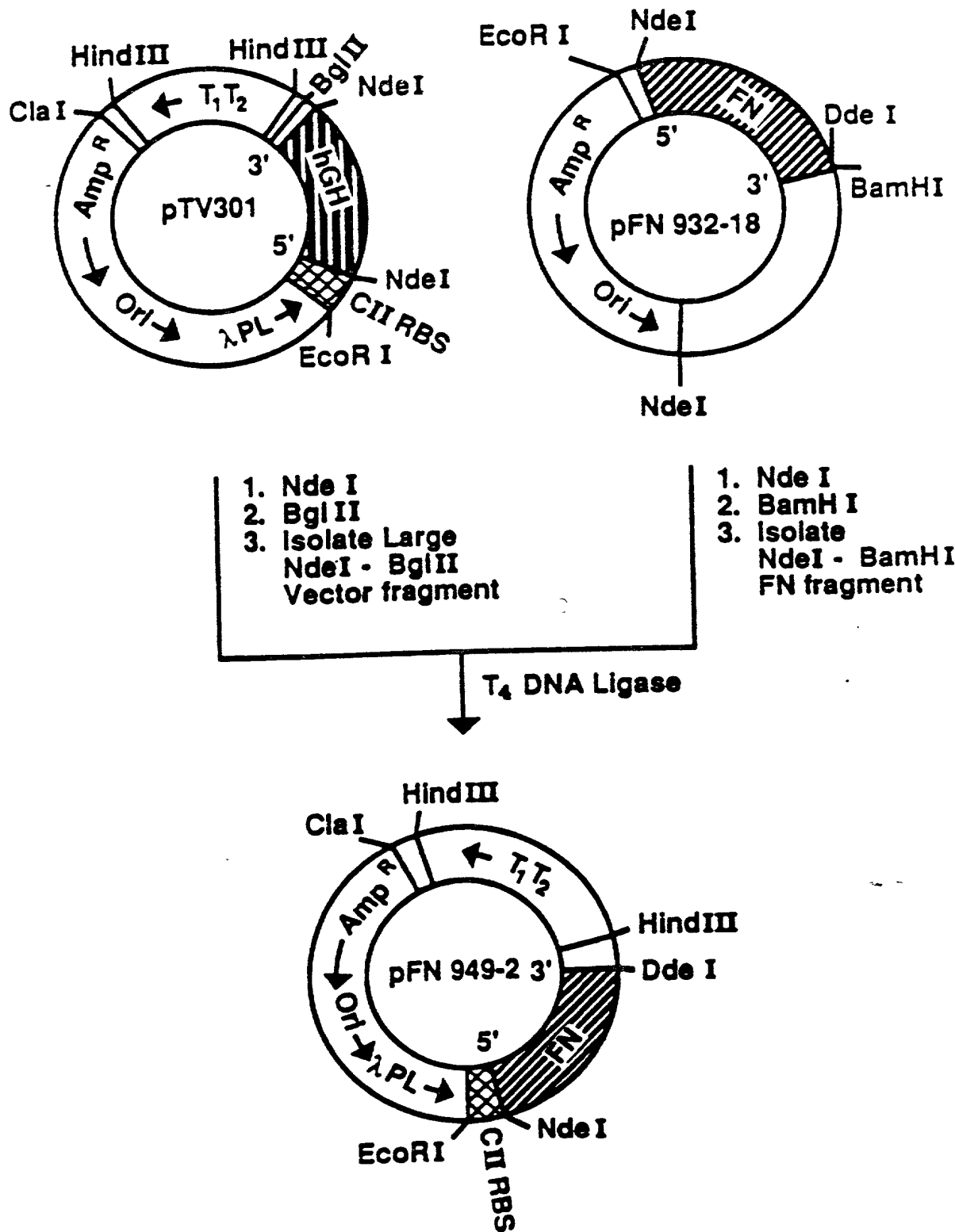


Figure 4



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Figure 5

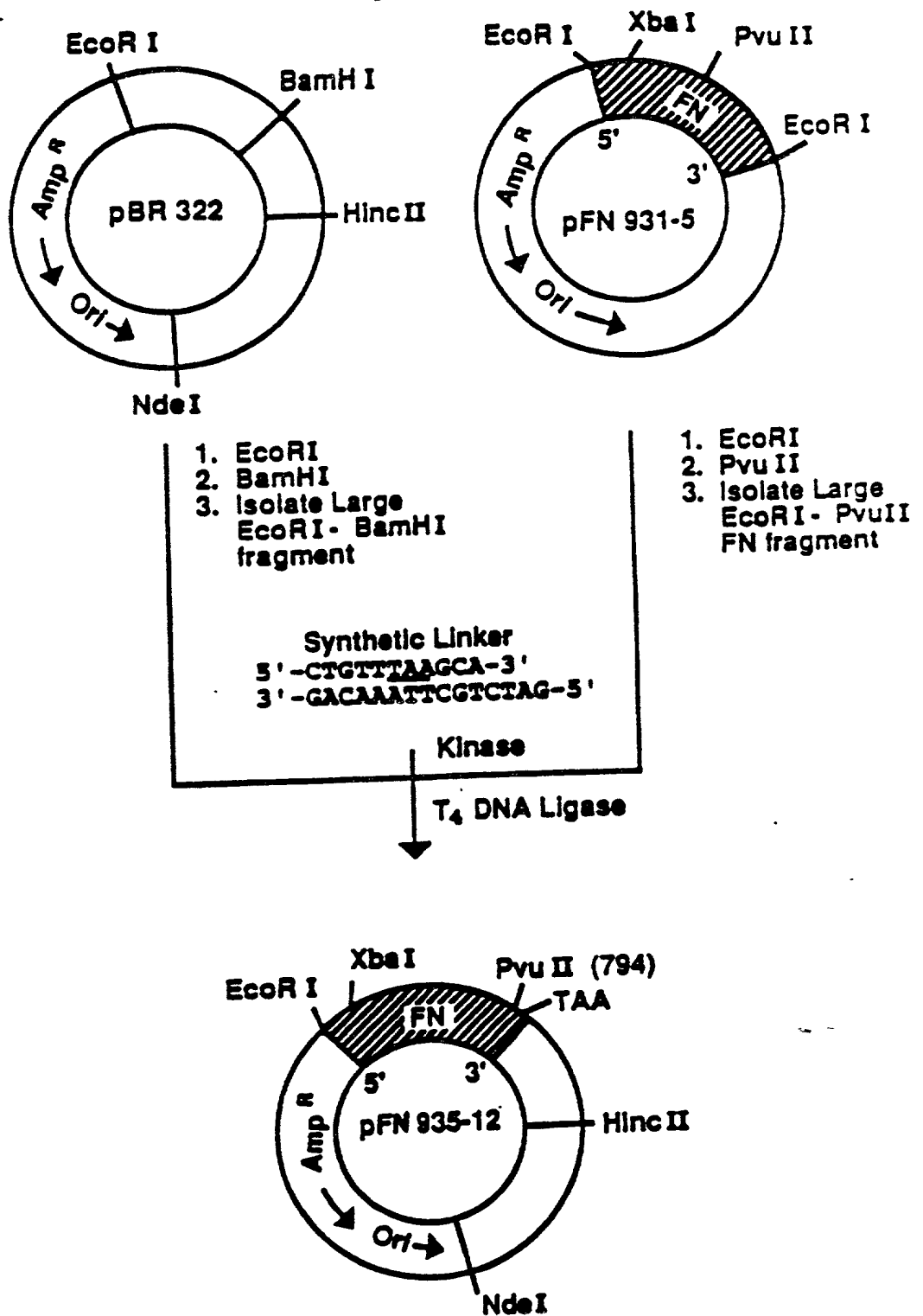
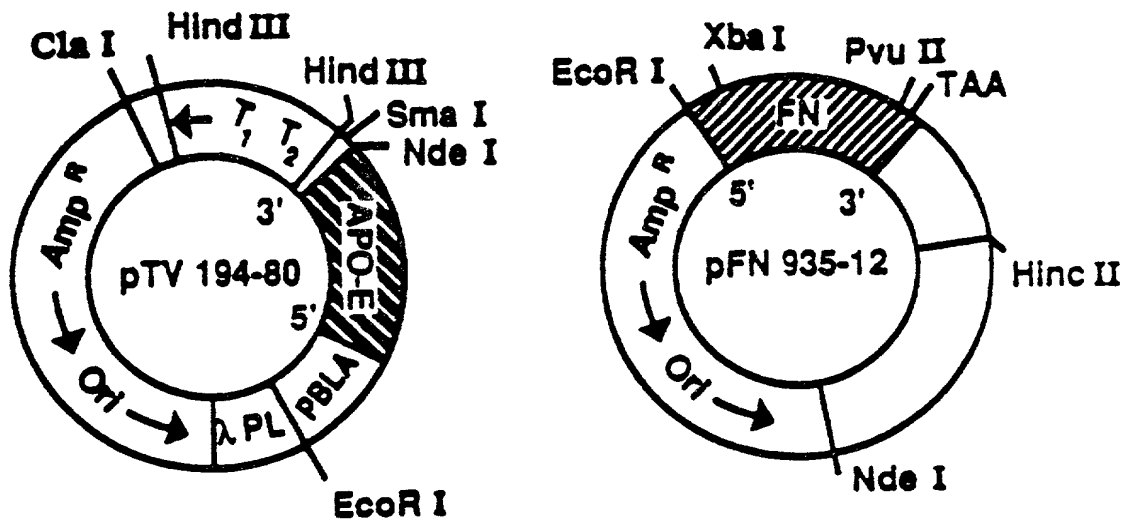


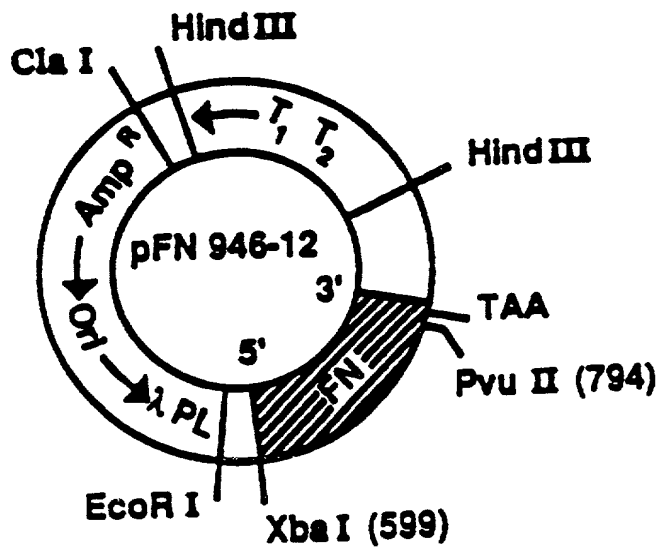
Figure 6



1. EcoR I
2. Sma I
3. Isolate large
EcoR I - Sma I fragment

1. EcoR I
2. Hinc II
3. Isolate
EcoR I - Hinc II
FN fragment

T₄ DNA Ligase



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Figure 7

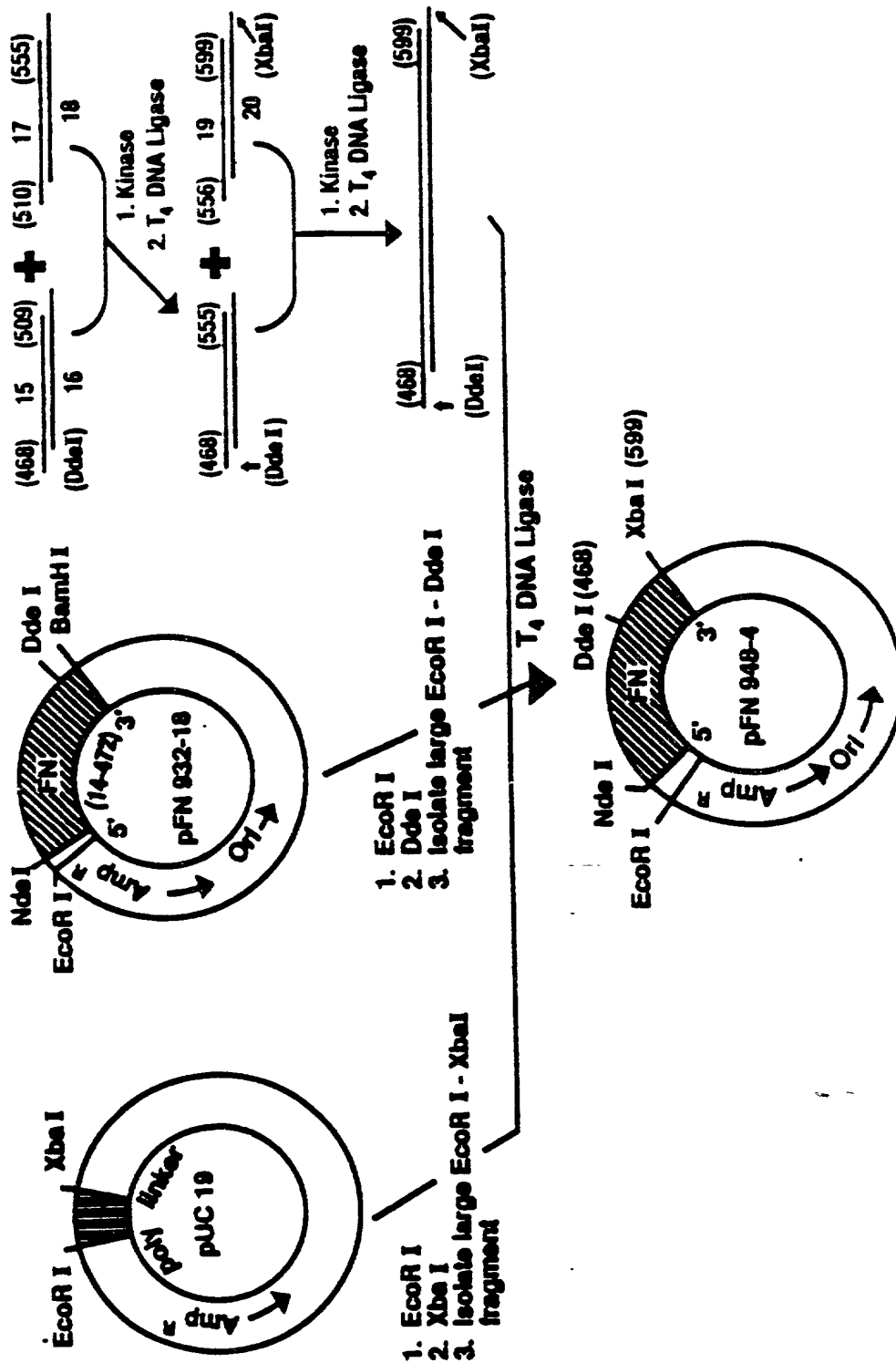


Figure 8

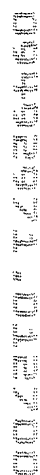


Figure 9

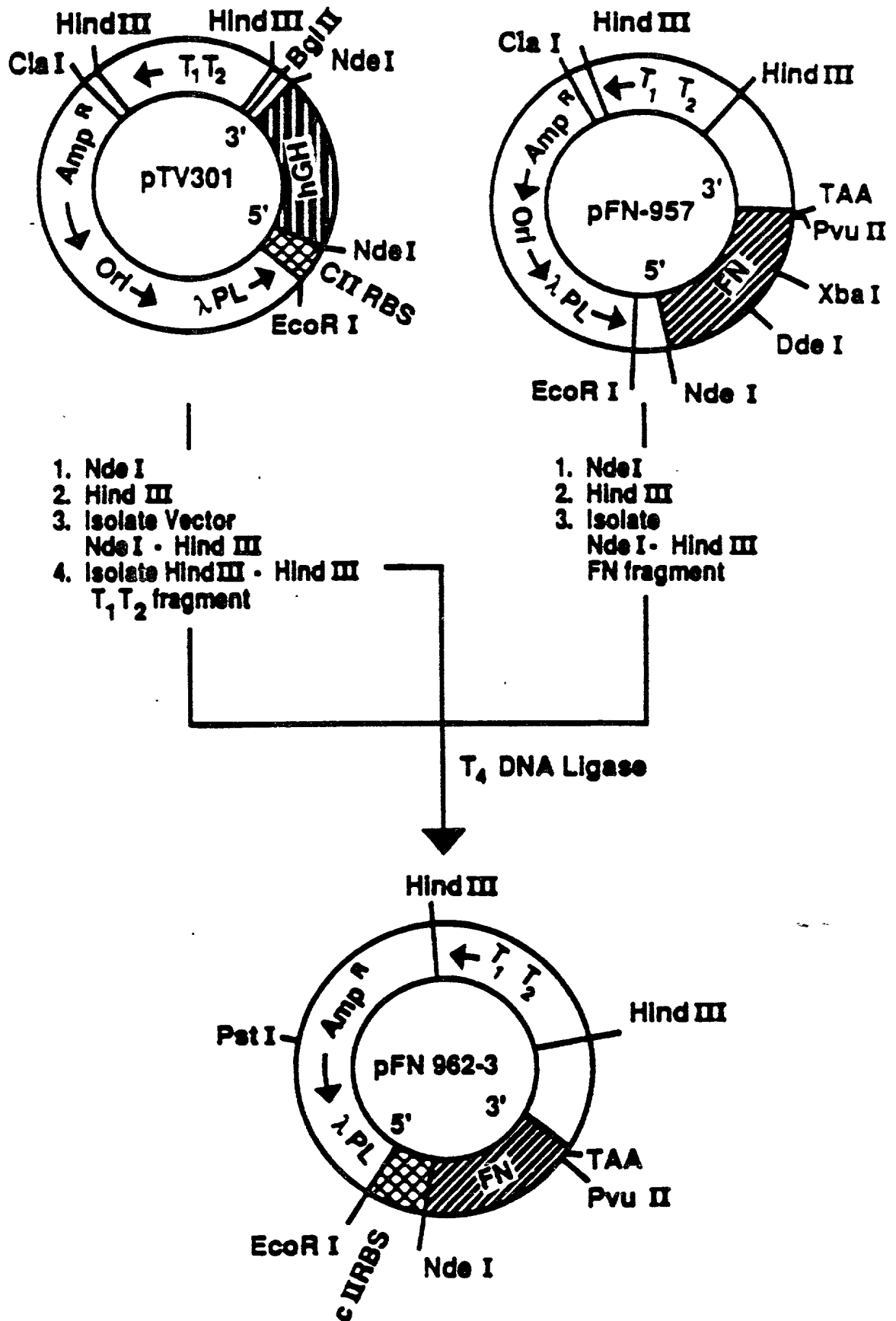
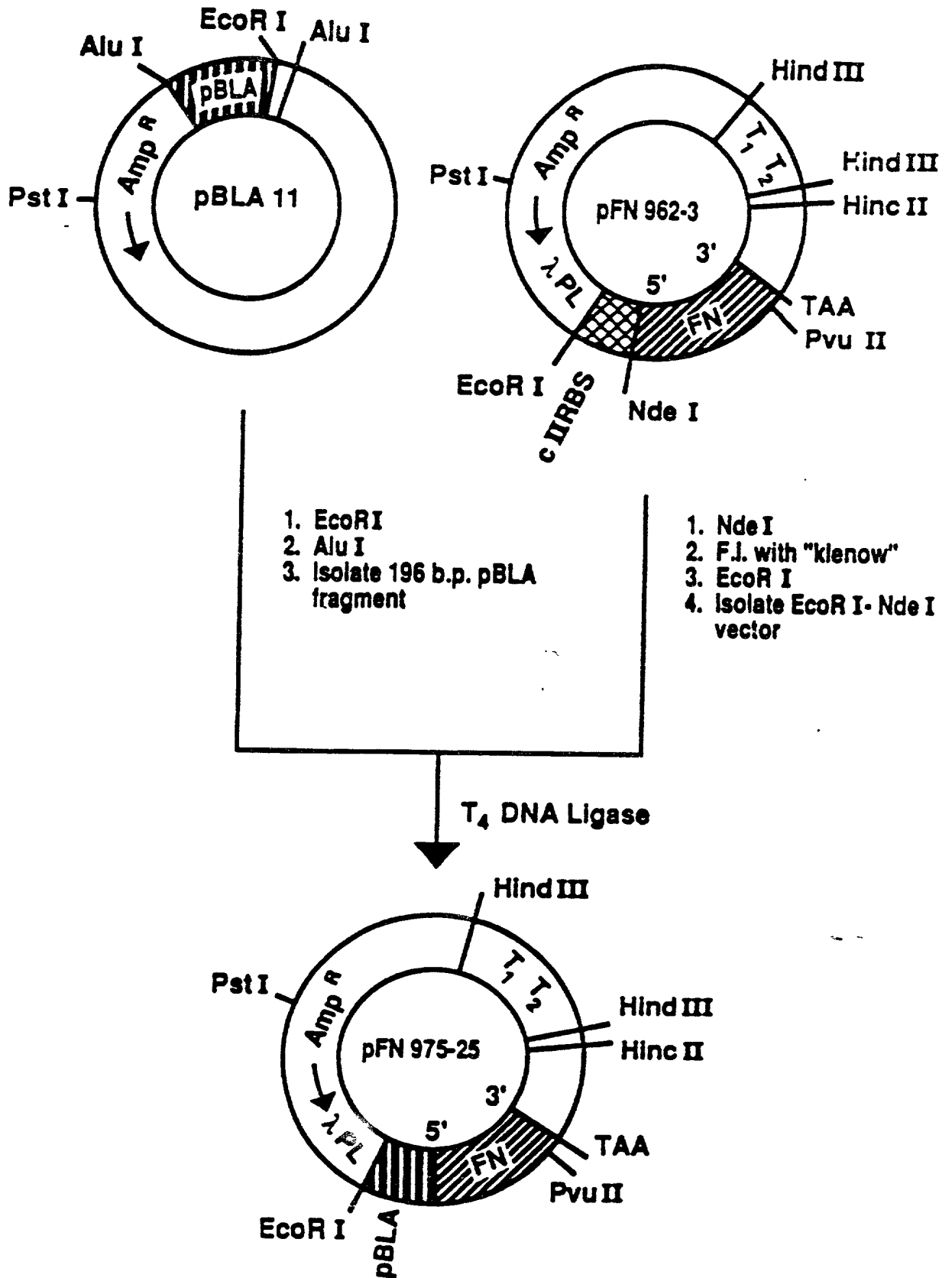


Figure 10



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Figure 11

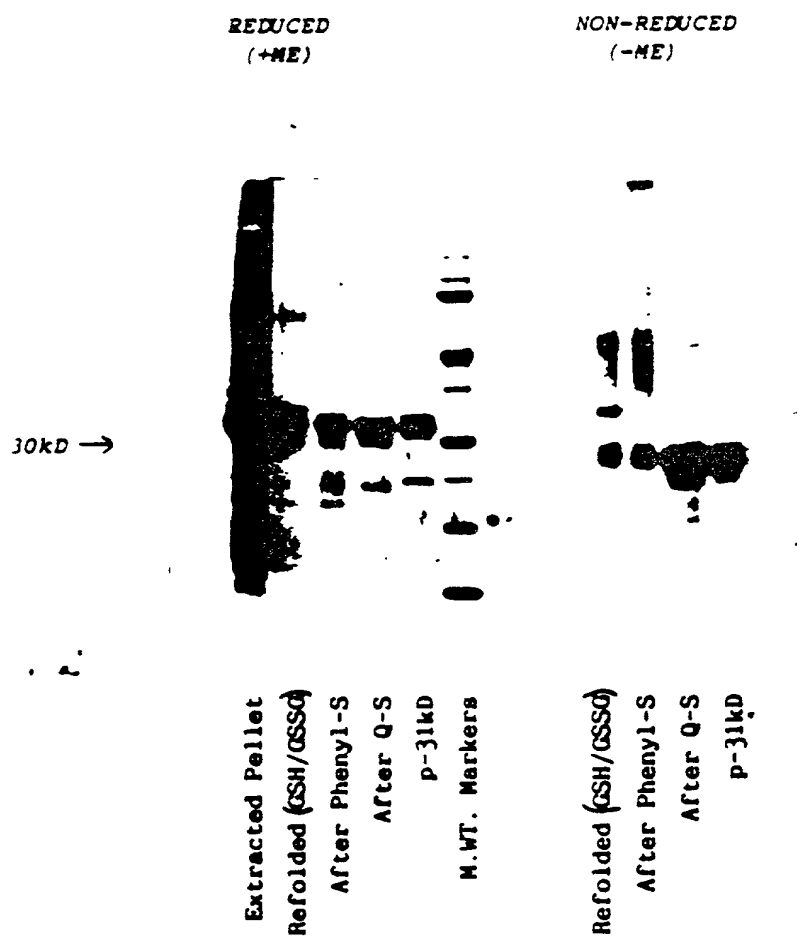


Figure 12

31kD / Phenyl-Sepharose

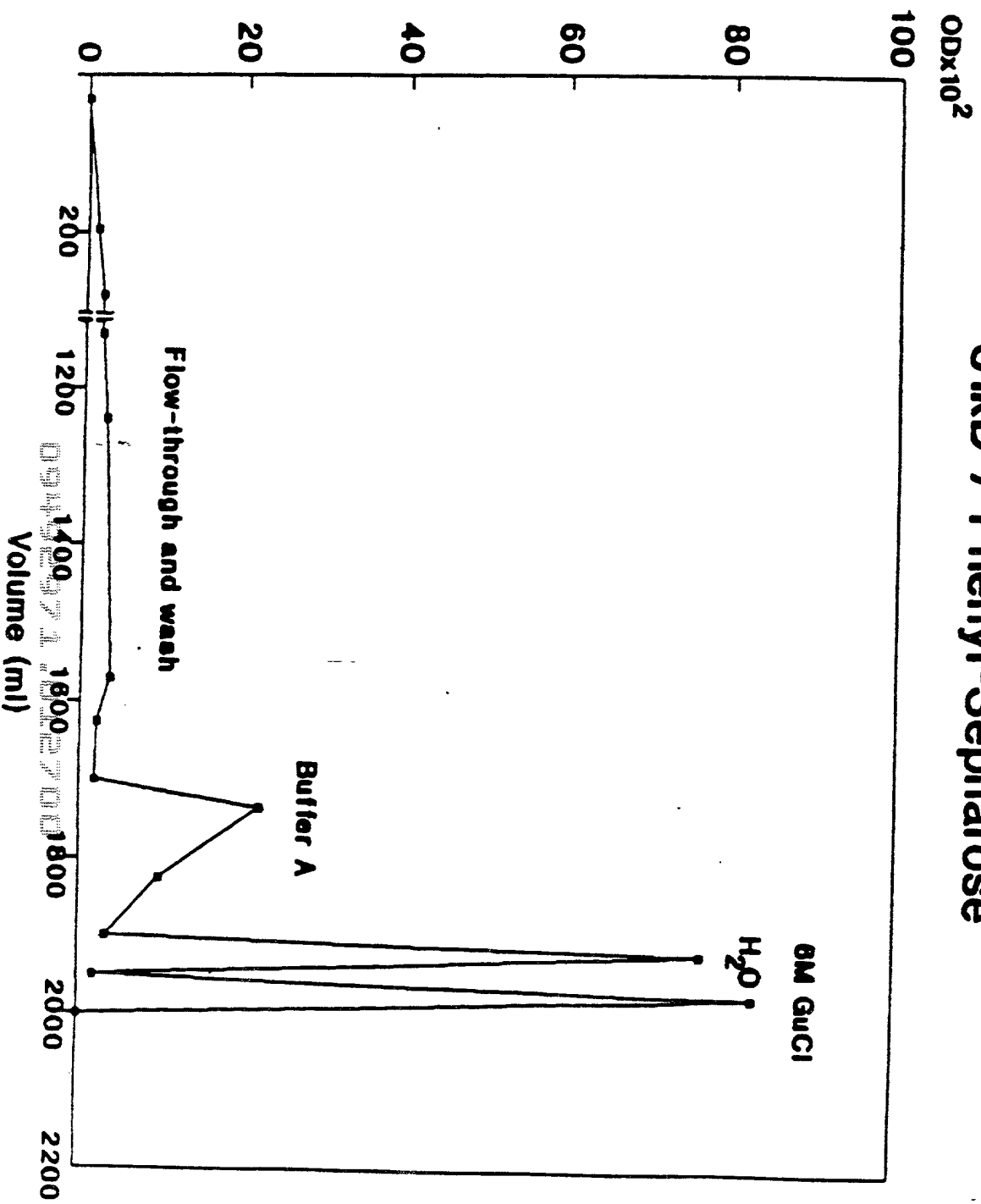
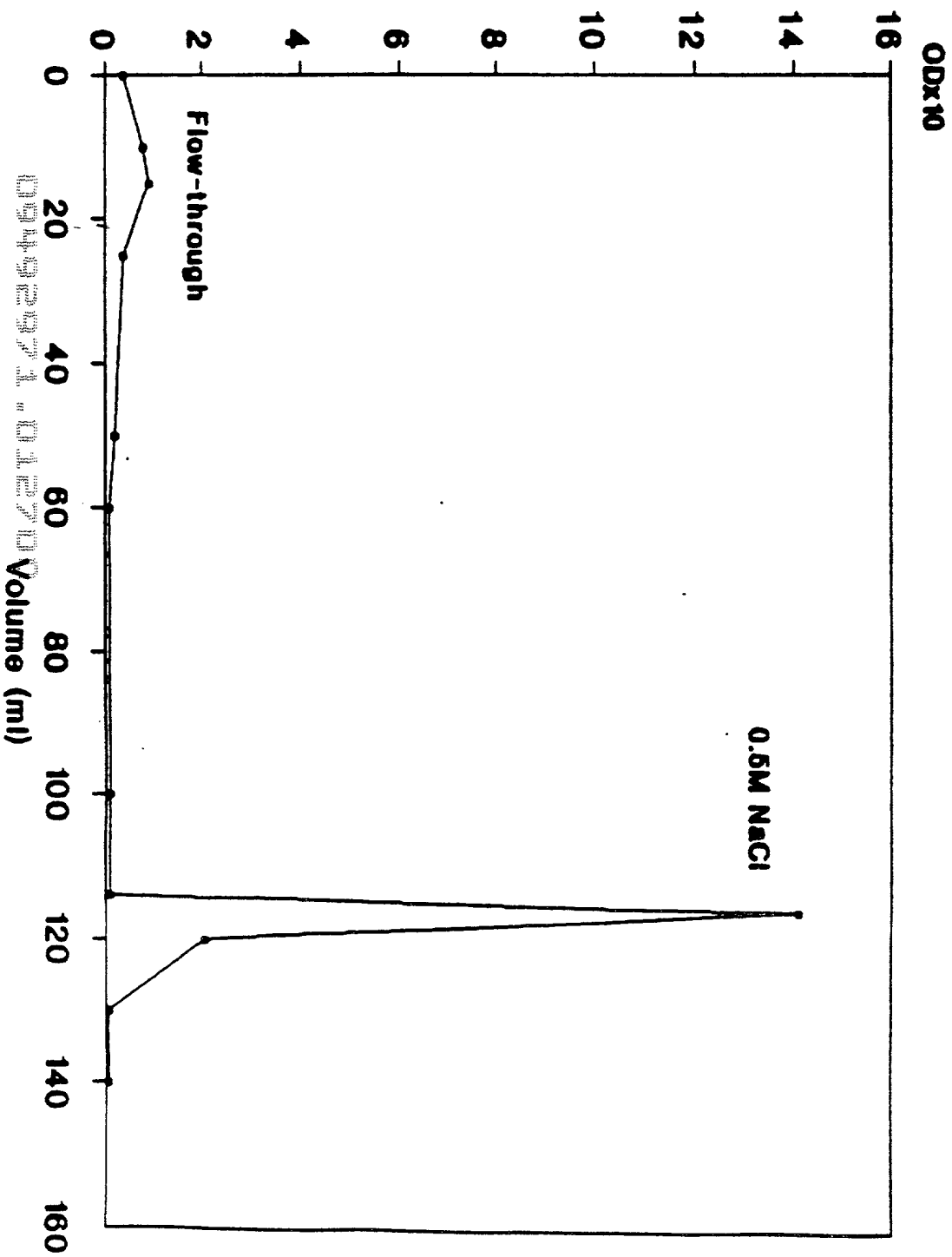


Figure 13

31kD/Heparin-Sephrose



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Figure 14

31kD / Q-Sepharose

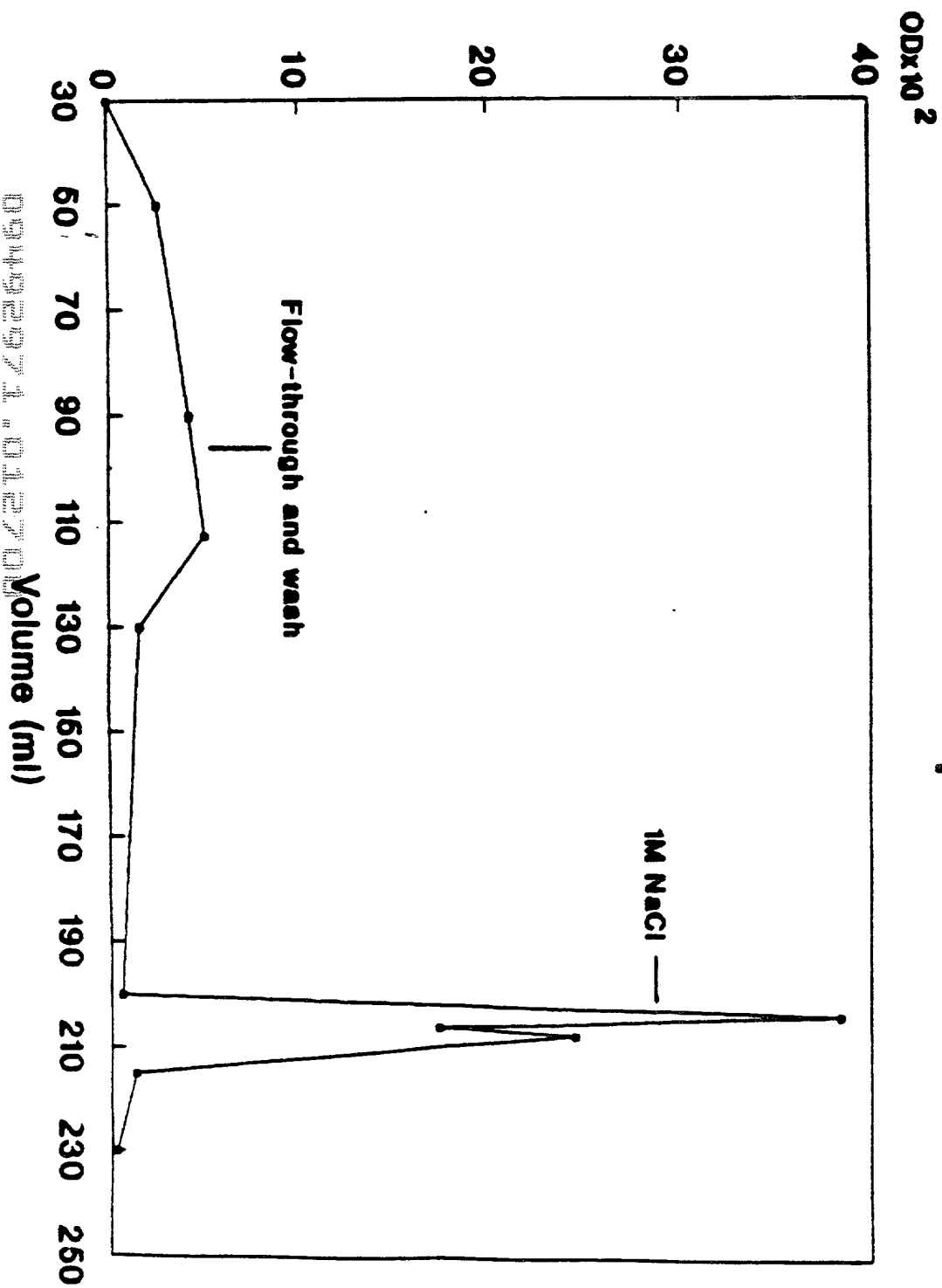
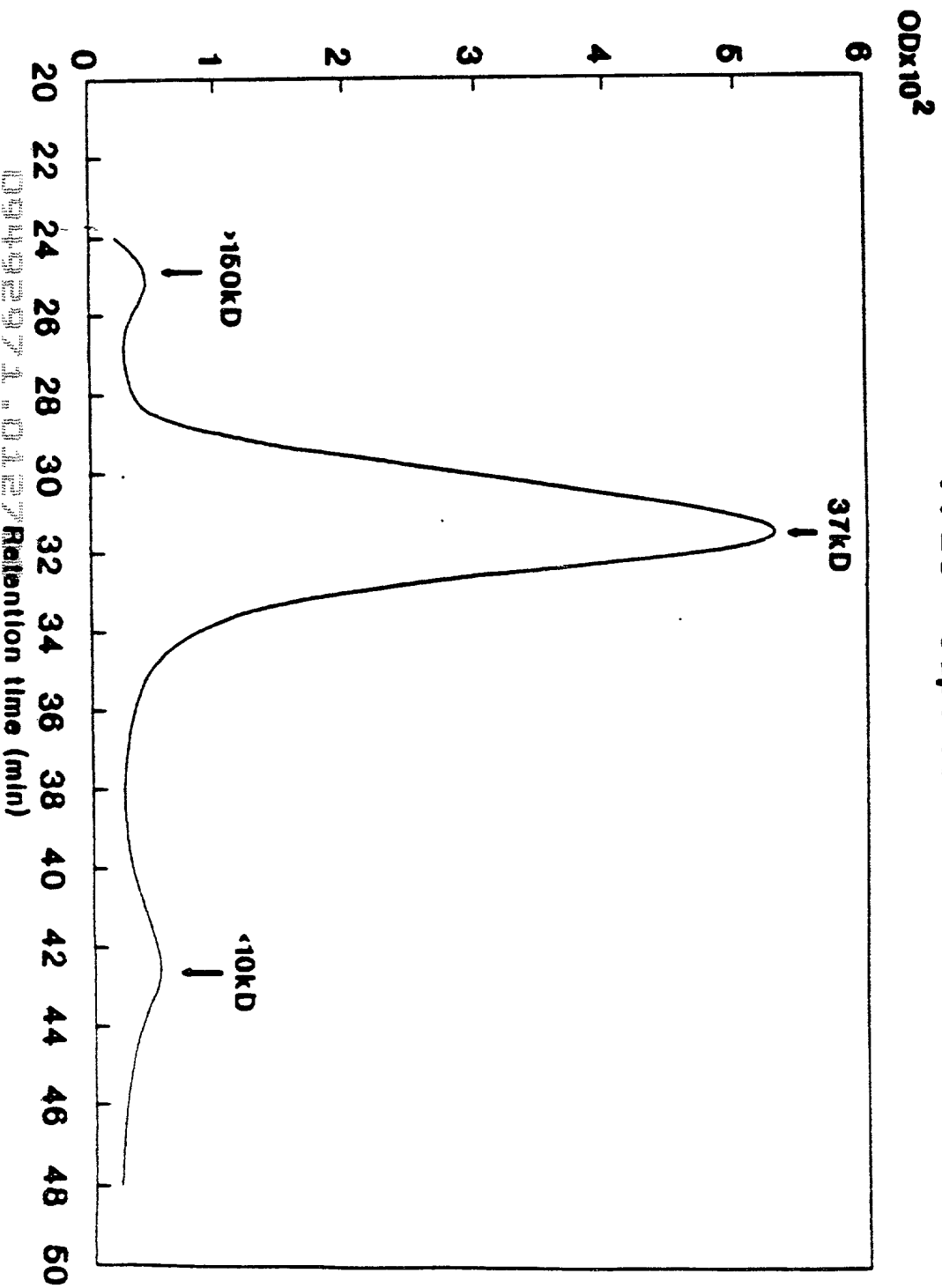


Figure 15

Mixture of rec. and plasmatic "31kD" FBD
FPLC - Superose 12



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Figure 16

Pharmacokinetic Behavior of Fibronectin
and Recombinant 31kD FBD in the Rat

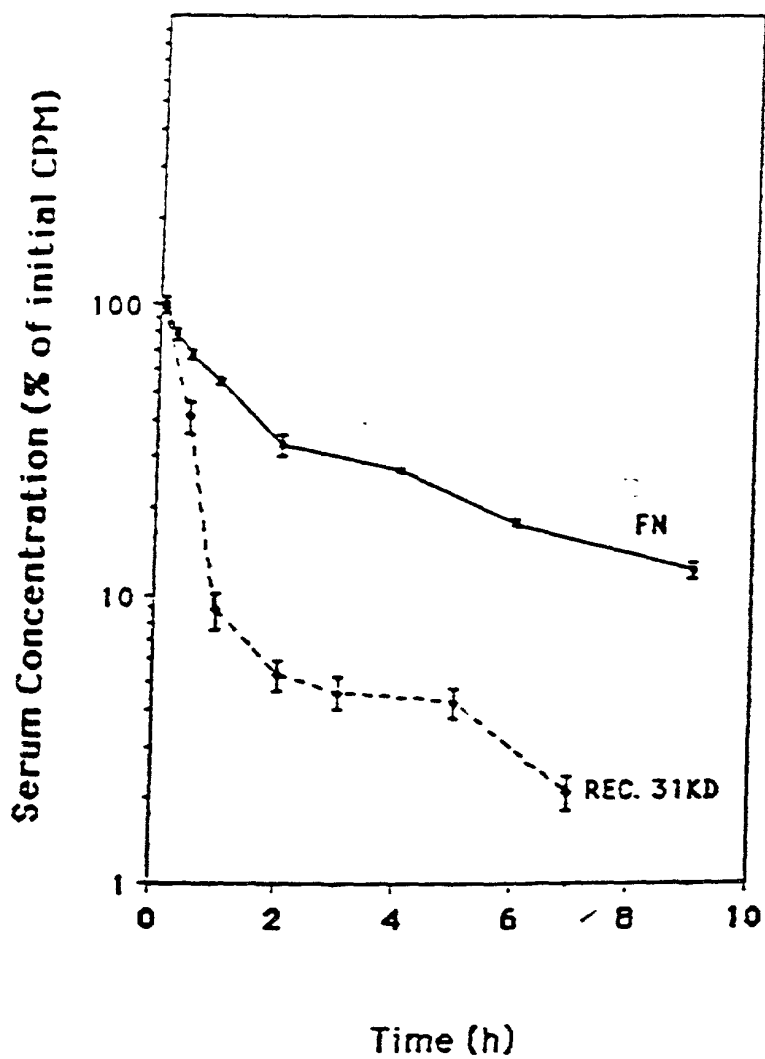
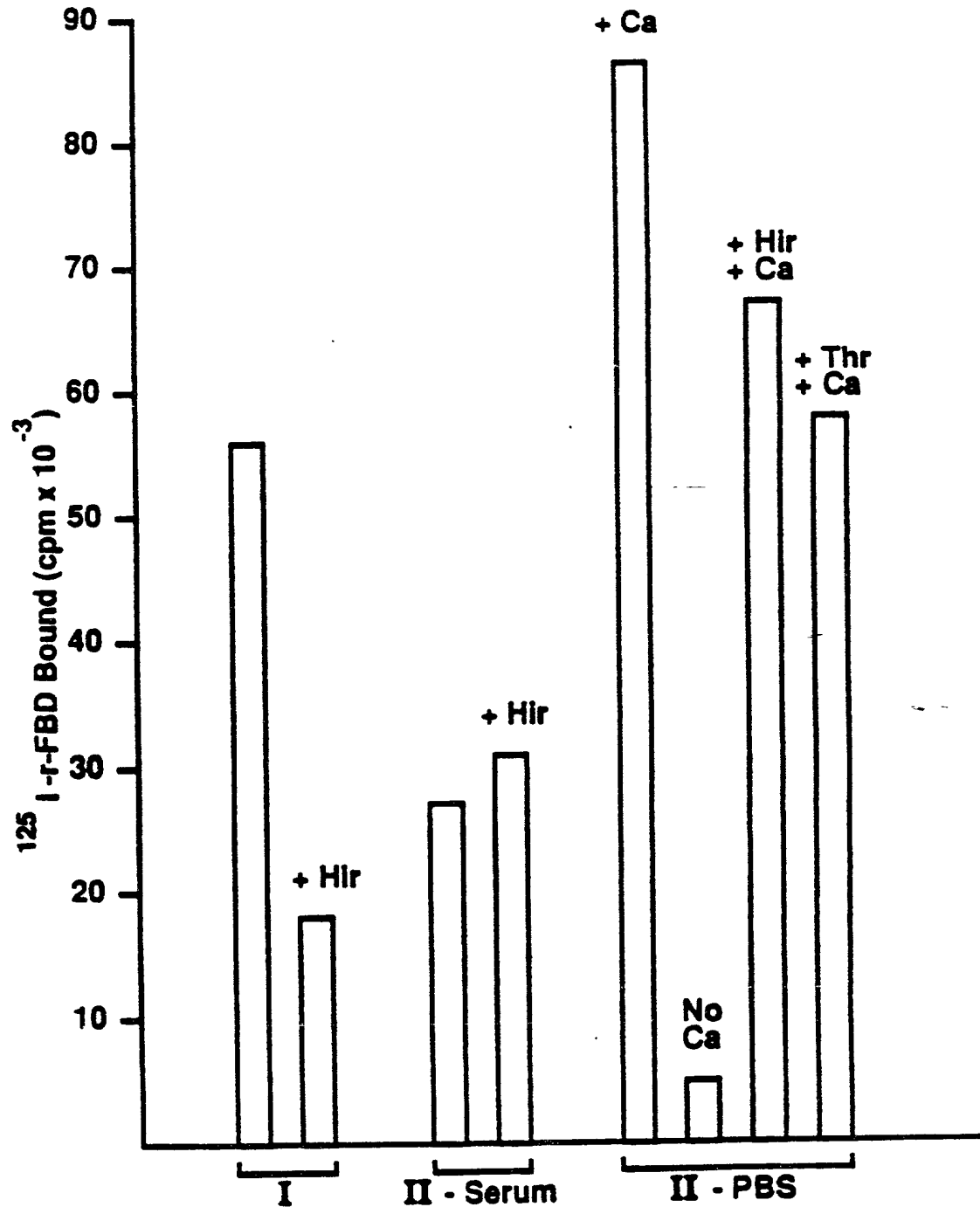


Figure 17

Binding of ^{125}I -FBD to Fibrin;
Effect of Thrombin and Ca^{++} ions.

I = Binding while clot formation

II = Binding to preformed Fibrin clot

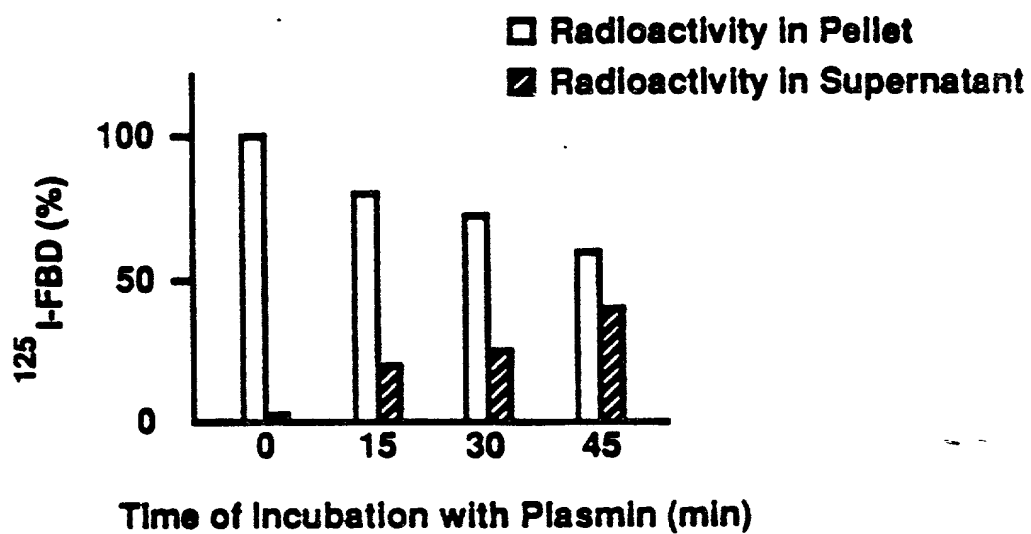


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Figure 18

Release of ^{125}I -FBD from Fibrin Clot by Plasmin.



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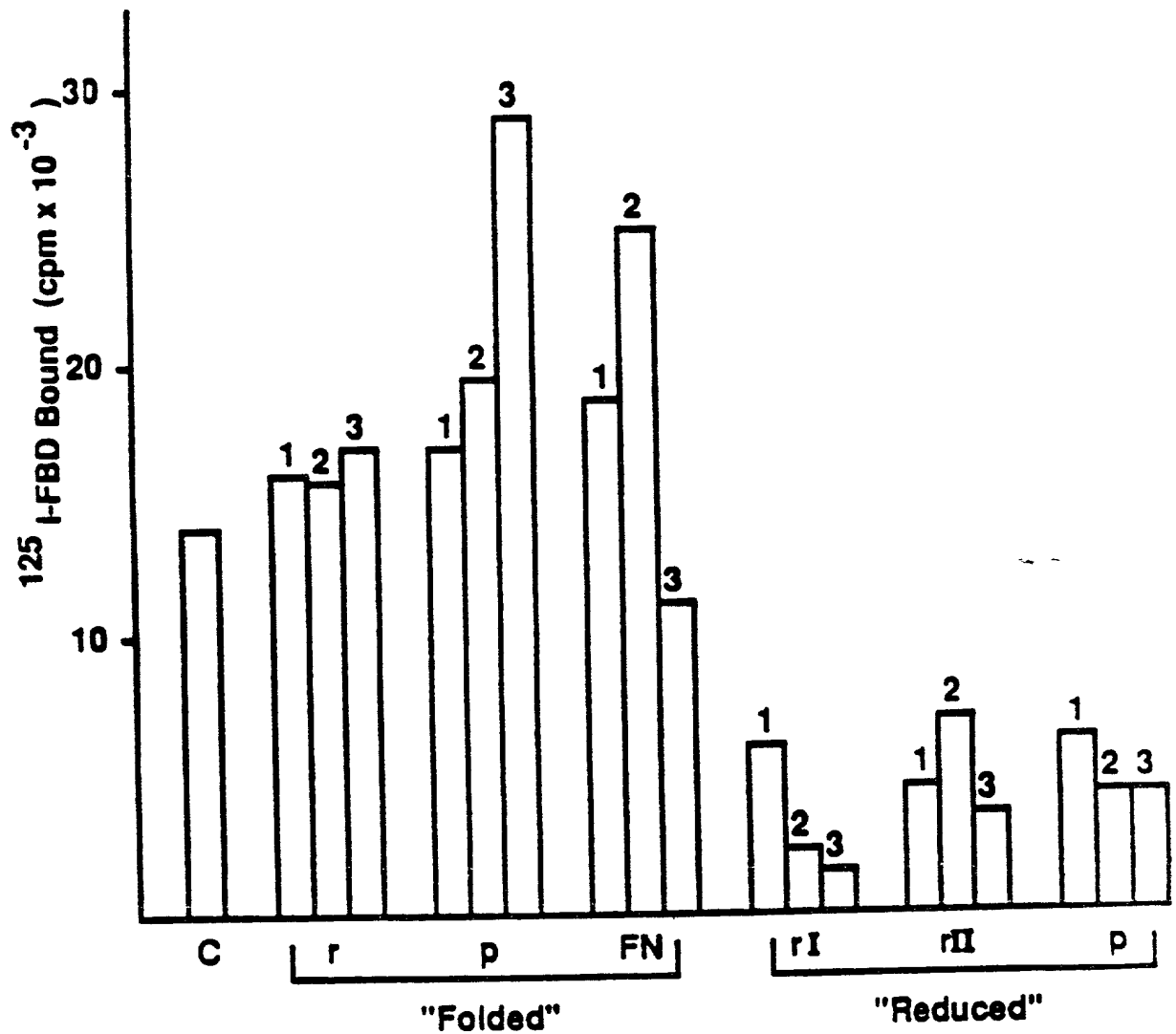
Figure 19

Binding of ^{125}I -FBD during clot formation (Reaction I) ;
Effect of unlabelled FBD and related molecules.

1 - 0.3 μM Unlabelled competitors

2 - 1.0 μM " "

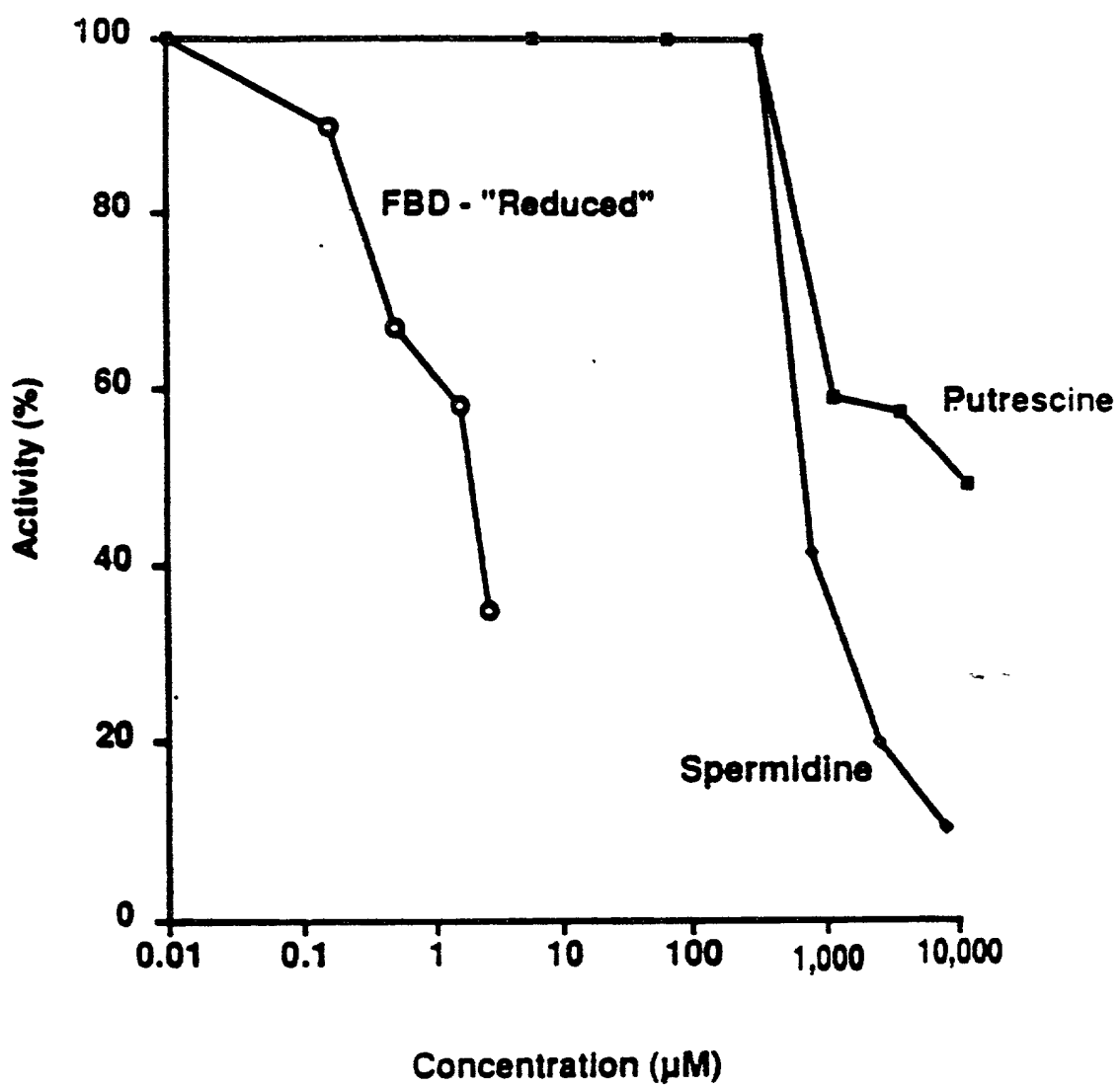
3 - 3.0 μM " "



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Figure 20

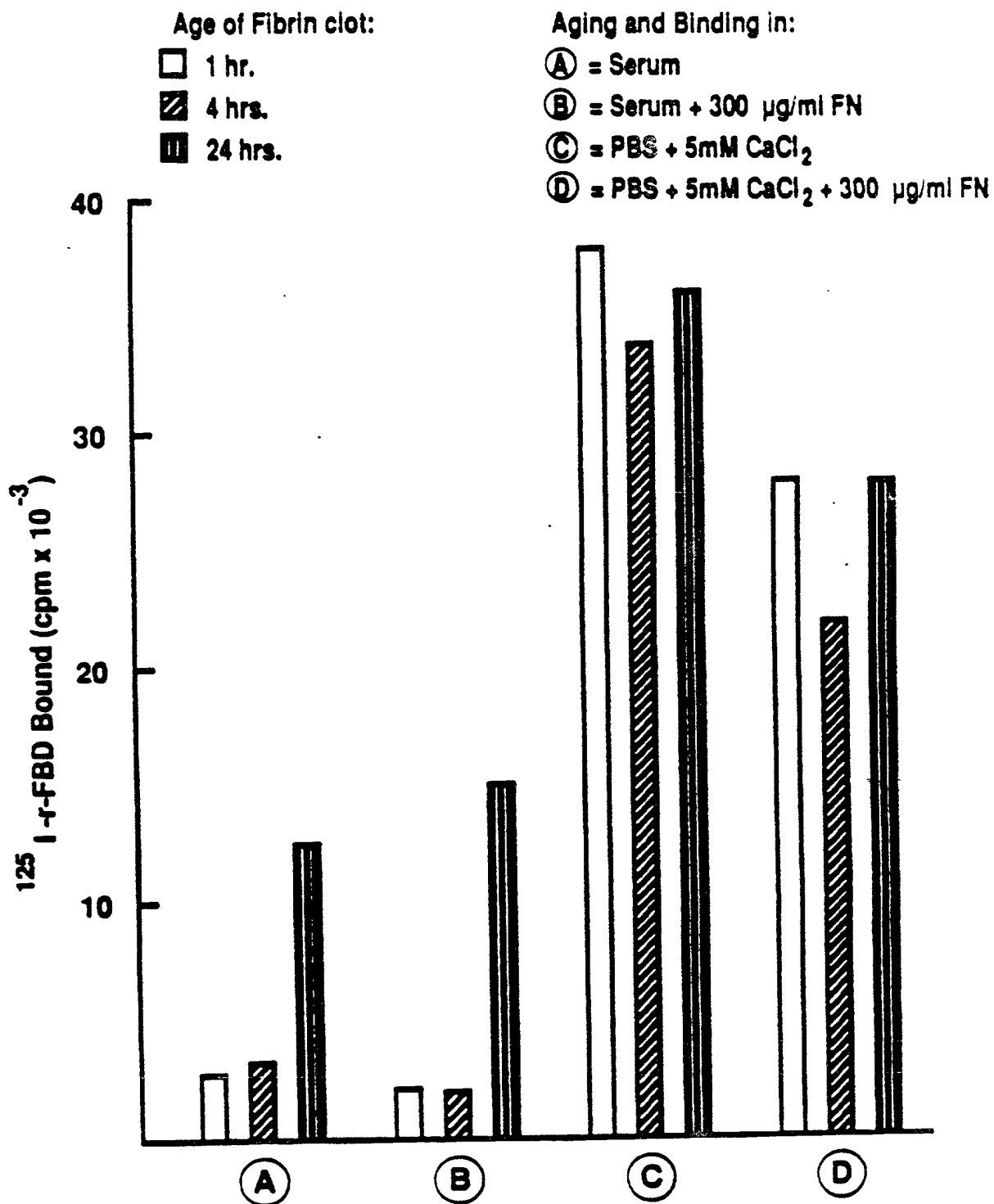
Binding of 125 I-FBD to Fibrin (Reaction II);
Effect of Transglutaminase Inhibitors.



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Figure 21

Binding of ^{125}I -FBD to preformed Fibrin clot (Reaction II);
Effect of Fibrin clot Age on the Binding (aging at 37° C).



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Figure 22

Binding of FBD to "Naive" Thrombi

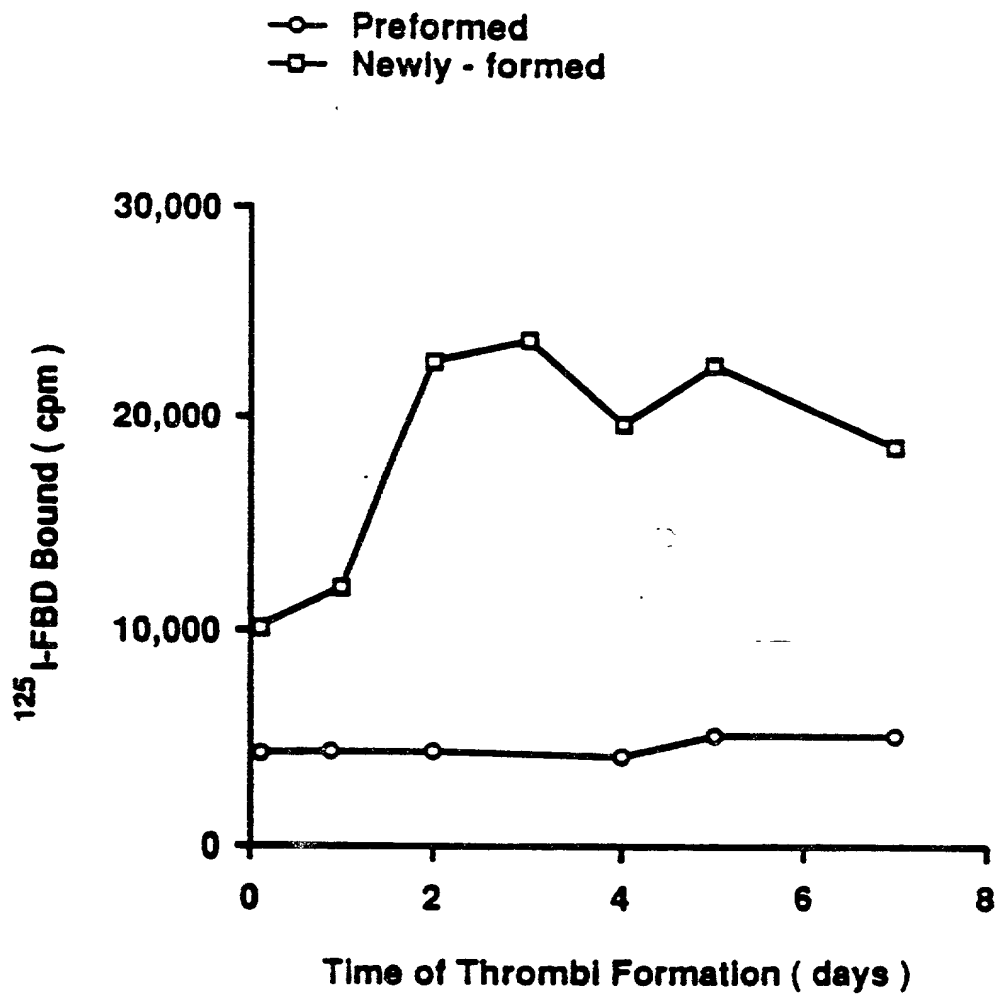


Figure 23

**Binding of FBD to Fibrin (Reaction I);
Effect of Transglutaminase ("TG")
and "Reduced" - FBD**

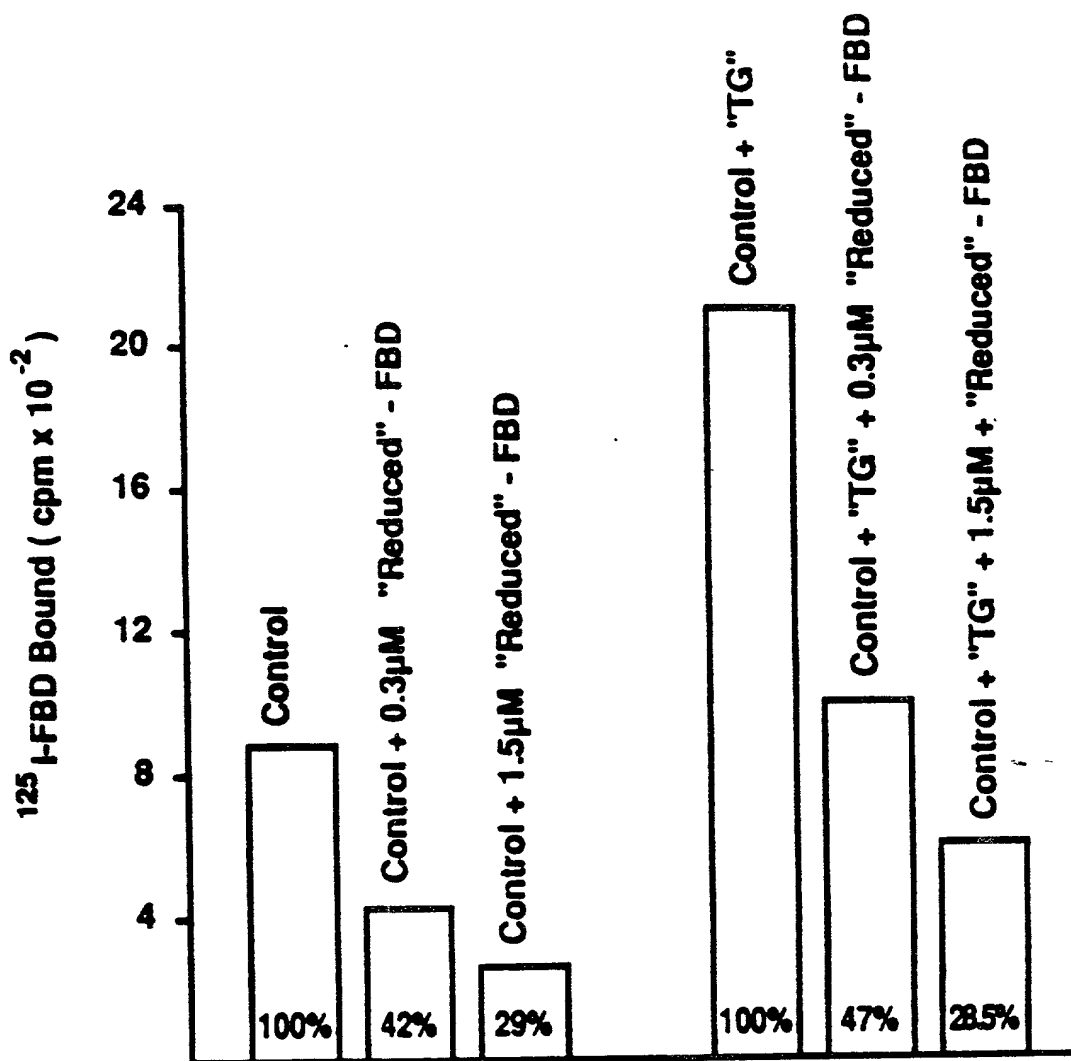


Figure 24

Binding of ^{125}I -FBD to ECM,
Effect of Ligand Concentration and Thrombin

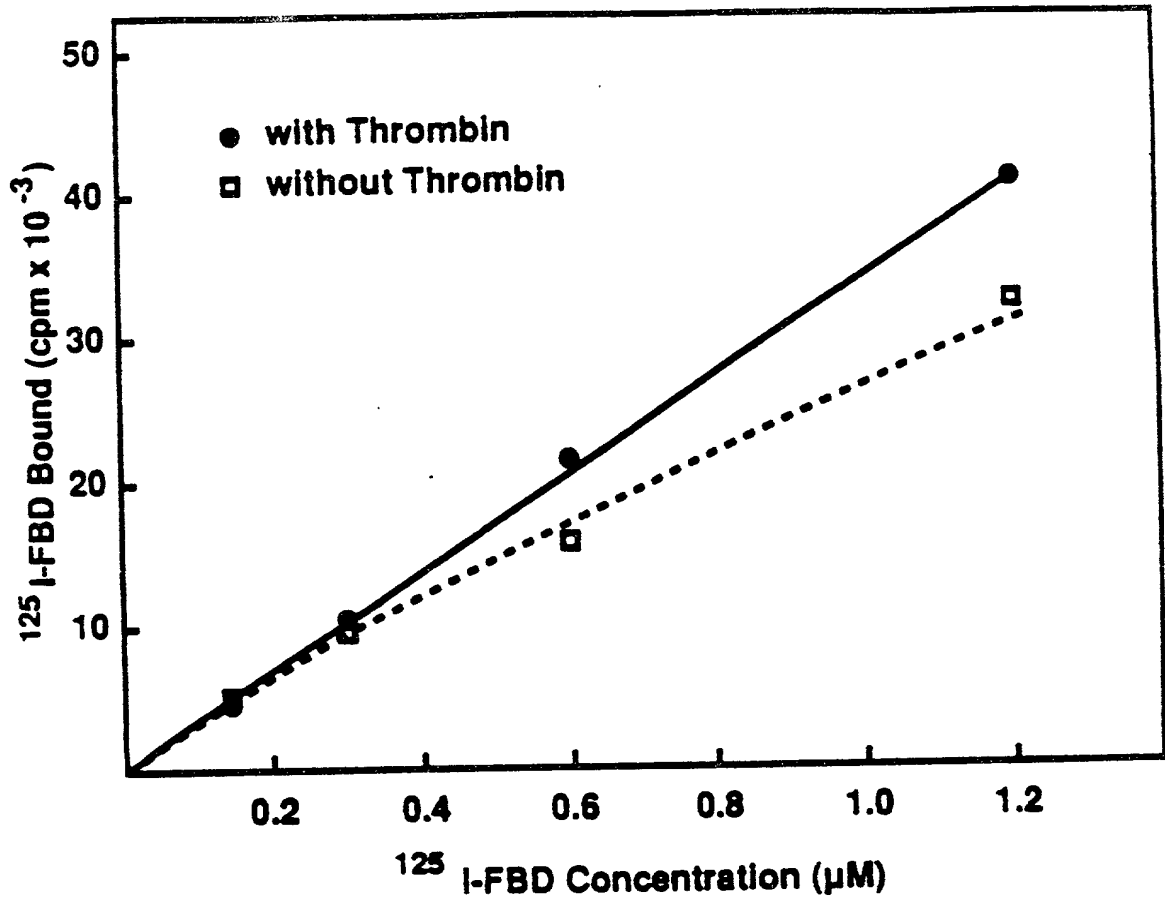


Figure 25

Binding of ^{125}I - (FN, r-FBD) to *S. aureus*

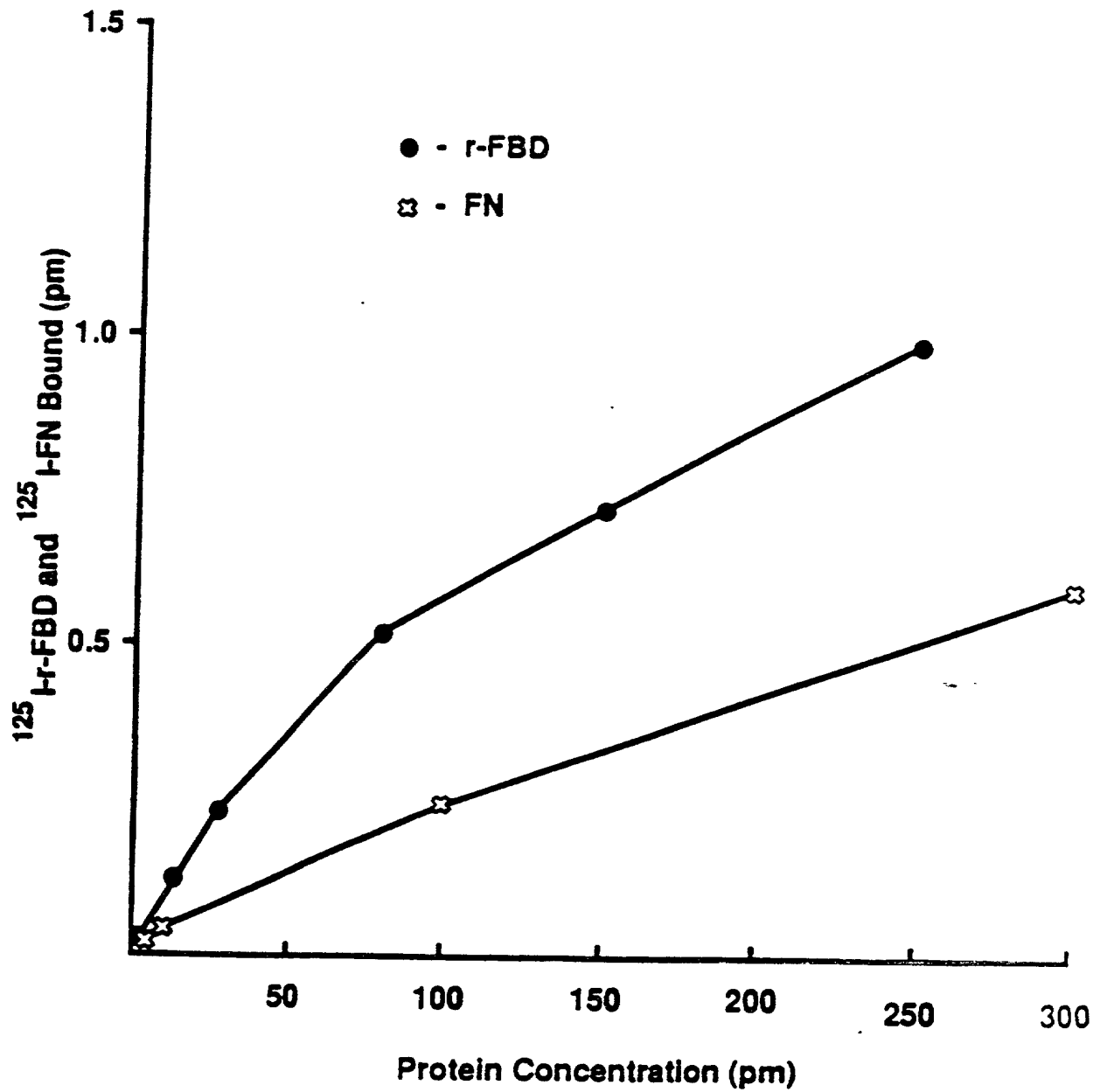


Figure 26

Binding of ^{125}I -FBD to *S. aureus*;
Competition with "Folded" and "Reduced" forms.

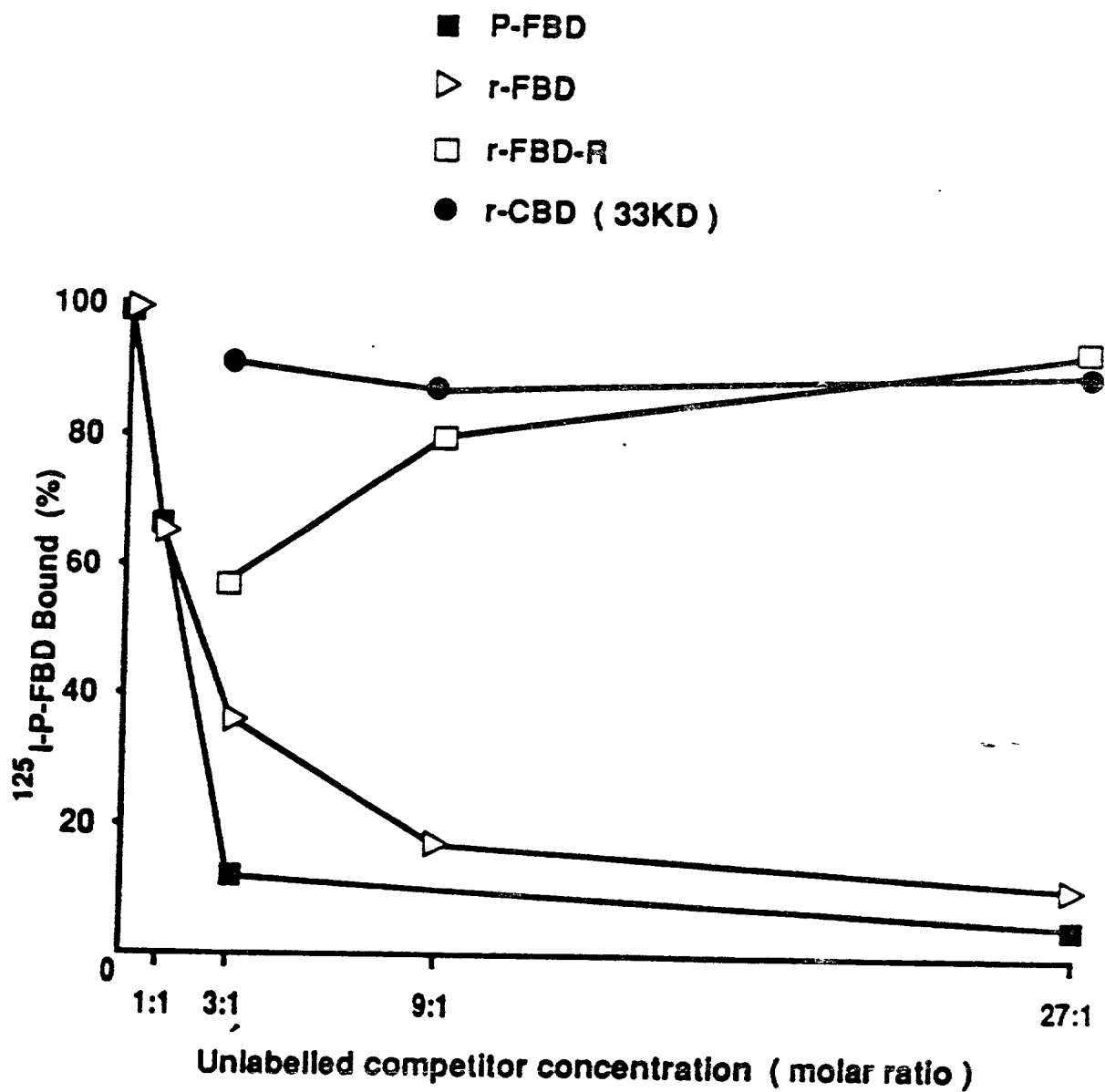


Figure 27

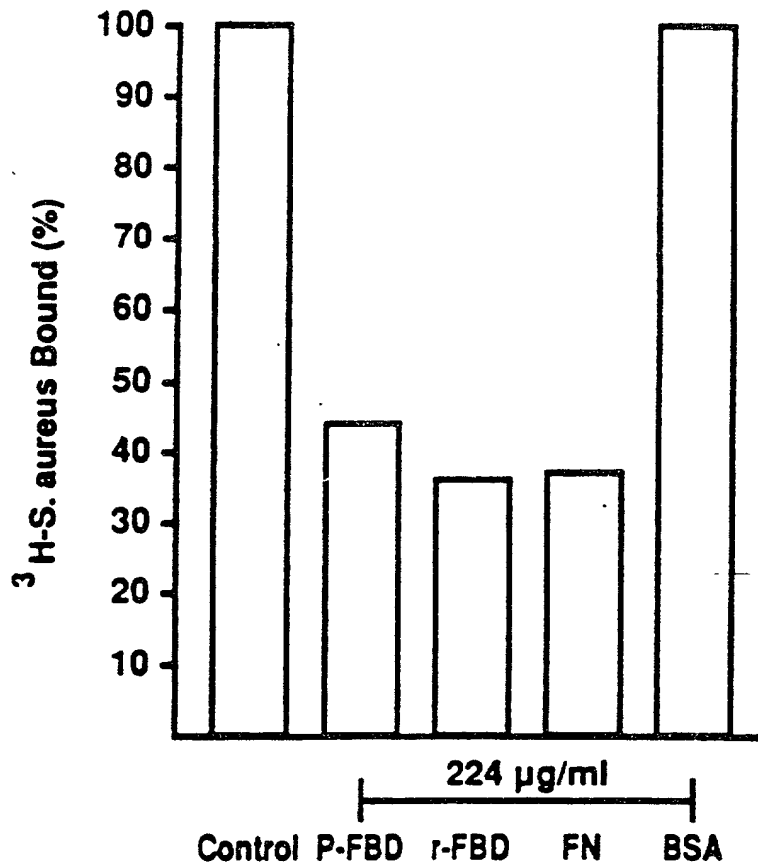
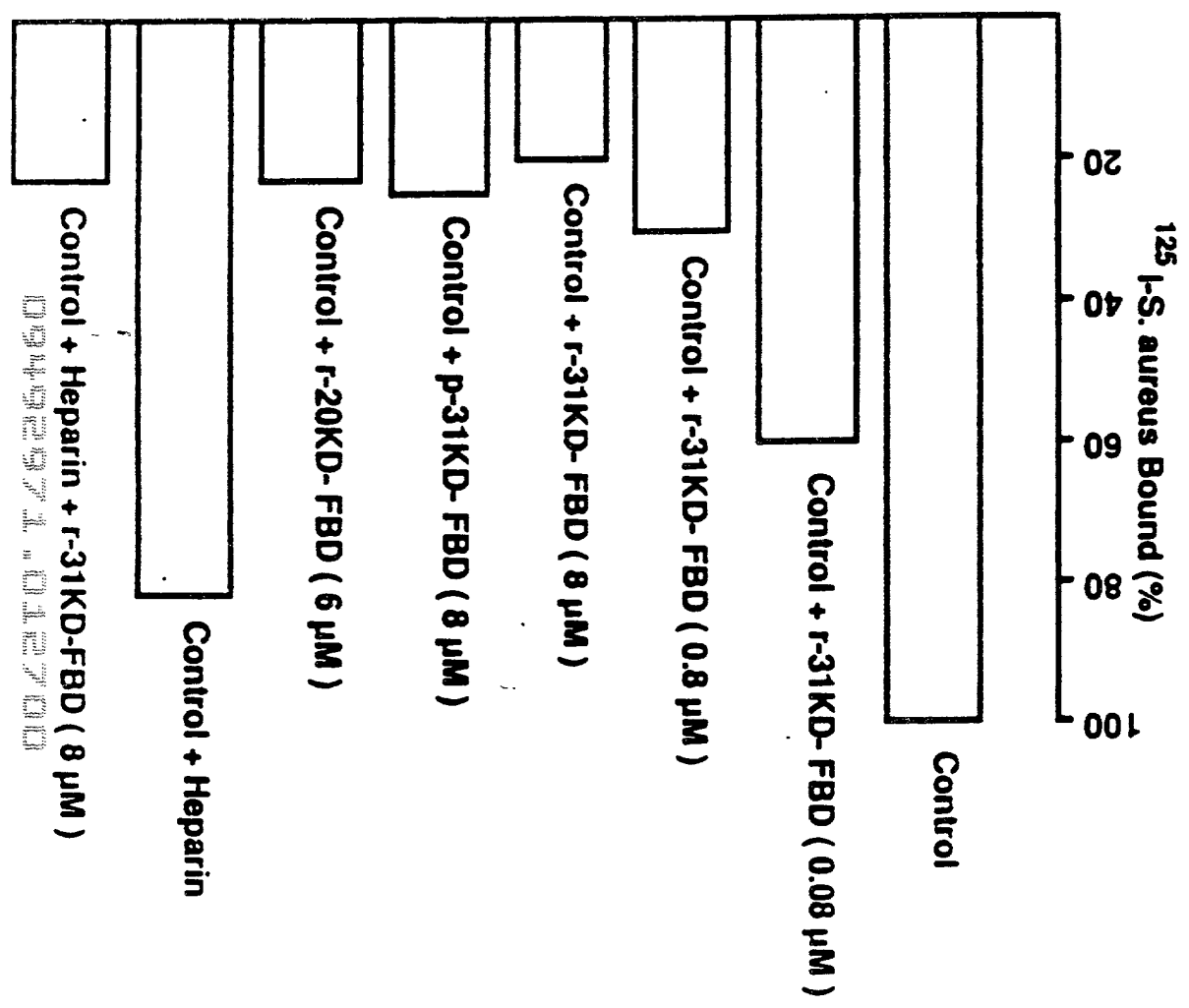
Binding of *S. aureus* to Immobilized FN.

Figure 28

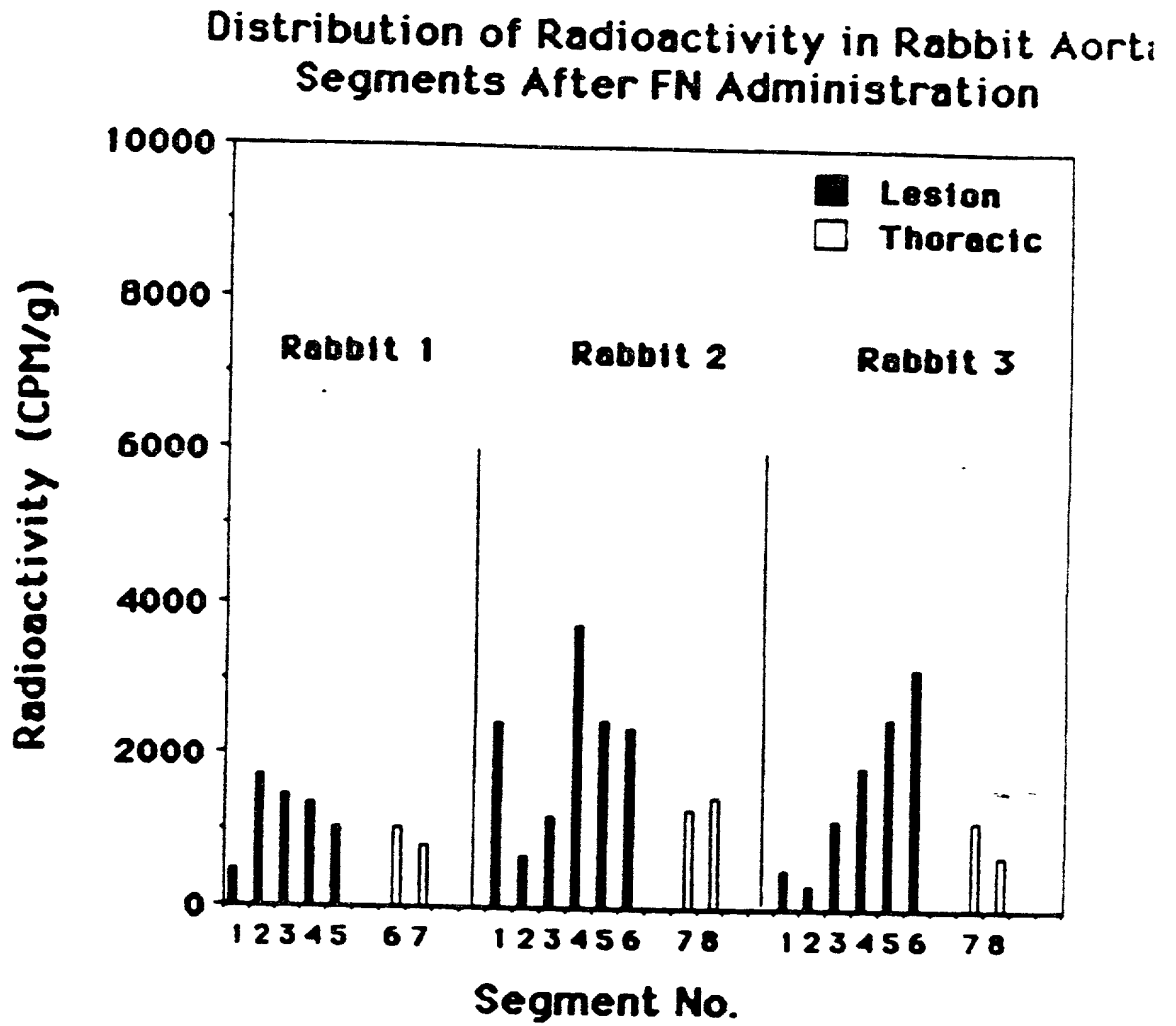
Binding of *S. aureus* to Bronchial Catheters;
Effect of FBD and Heparin.



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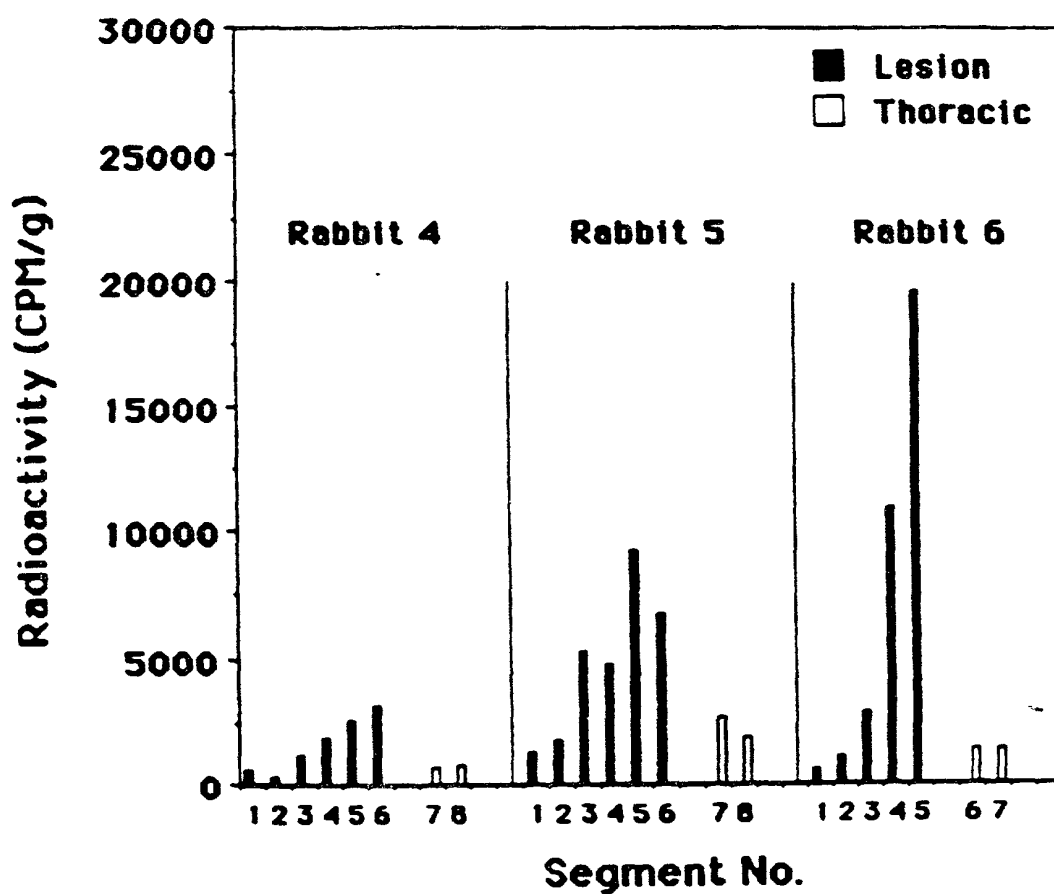
Figure 29A



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Figure 29B

**Distribution of Radioactivity in Rabbit Aorta
Segments After 31kD FBD Administration**



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Figure 30

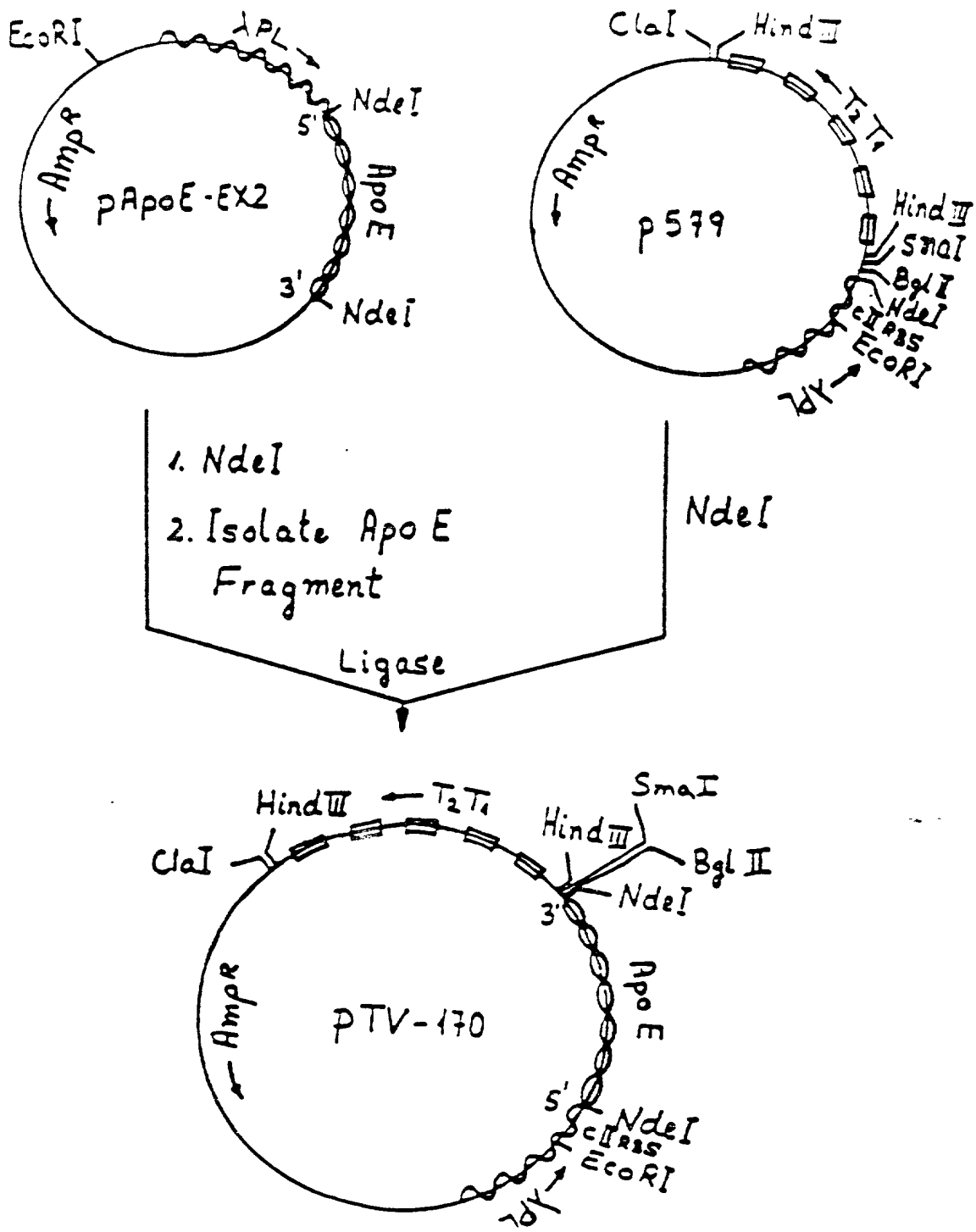
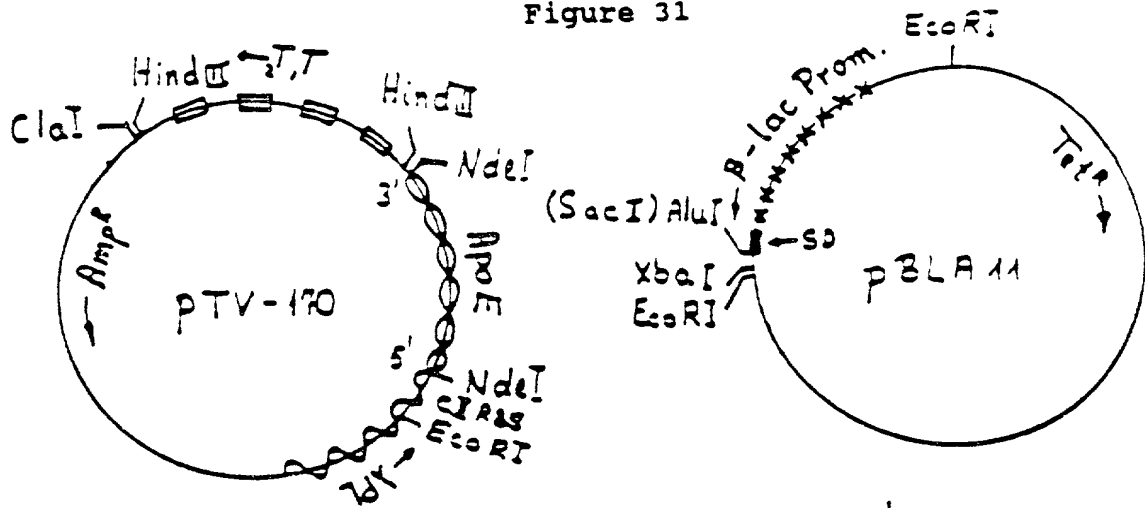
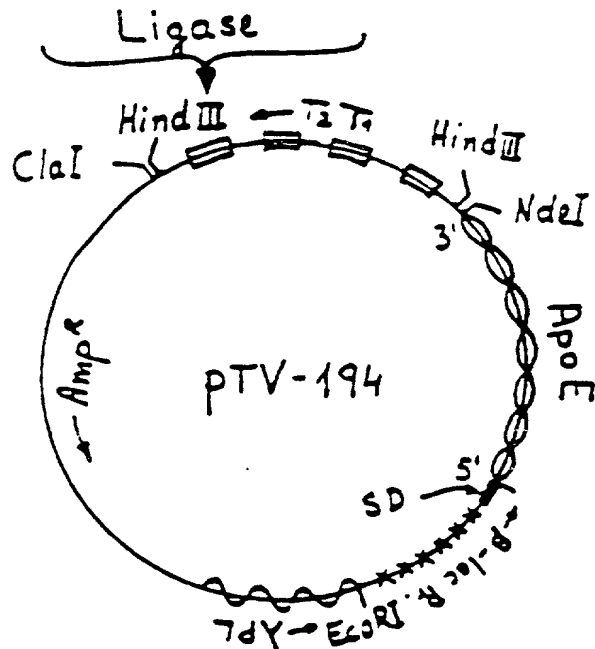
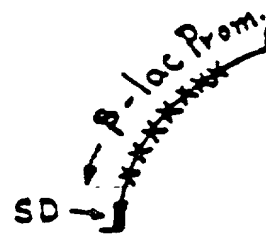
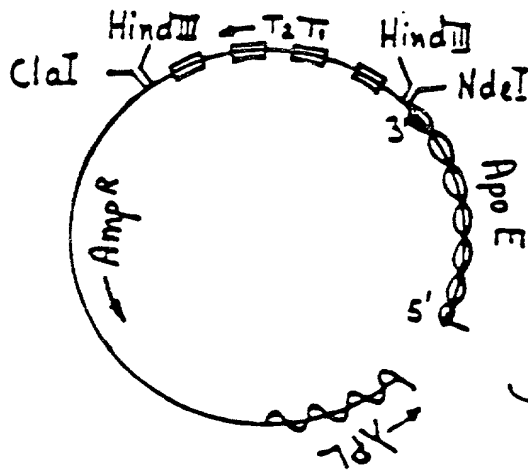


Figure 31



1. Partial NdeI
2. Fill in
3. EcoRI
4. Isolate Large Fragment

1. AluI+EcoRI
2. Isolate 2003. Fragment



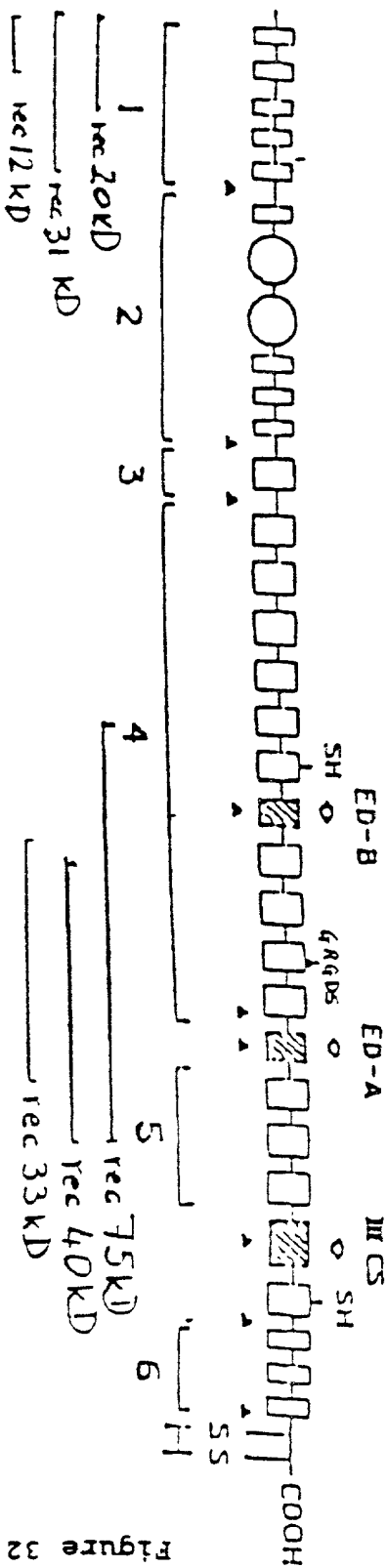
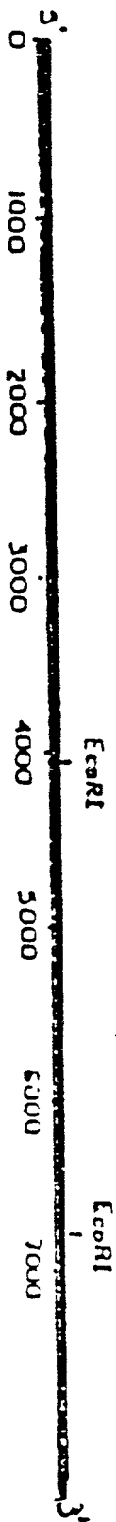


Figure 32
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HEPARIN COLLAGEN [HEPARIN]
S. AUREUS [DNA]
FIBRIN CELL
HEPARIN DNA
HEPARIN [CELL] FIBRIN

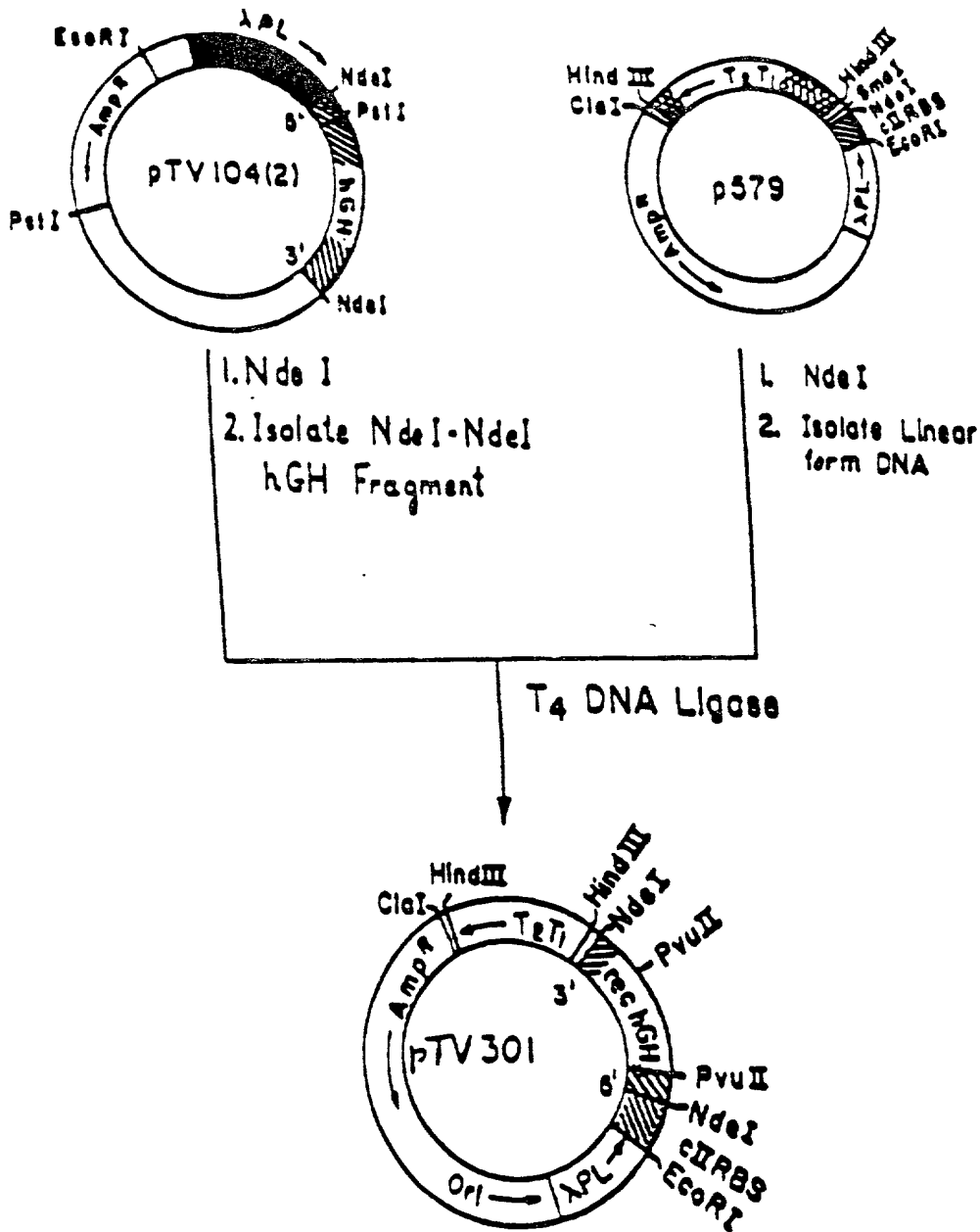
Nucleotides

rec-12kD:	14 - 340
rec-20kD:	14 - 432
rec-31kD:	14 - 799
rec-40kD:	4151 - 5566
rec-75kD:	2317 - 5566
rec-33kD:	3998 - 5179

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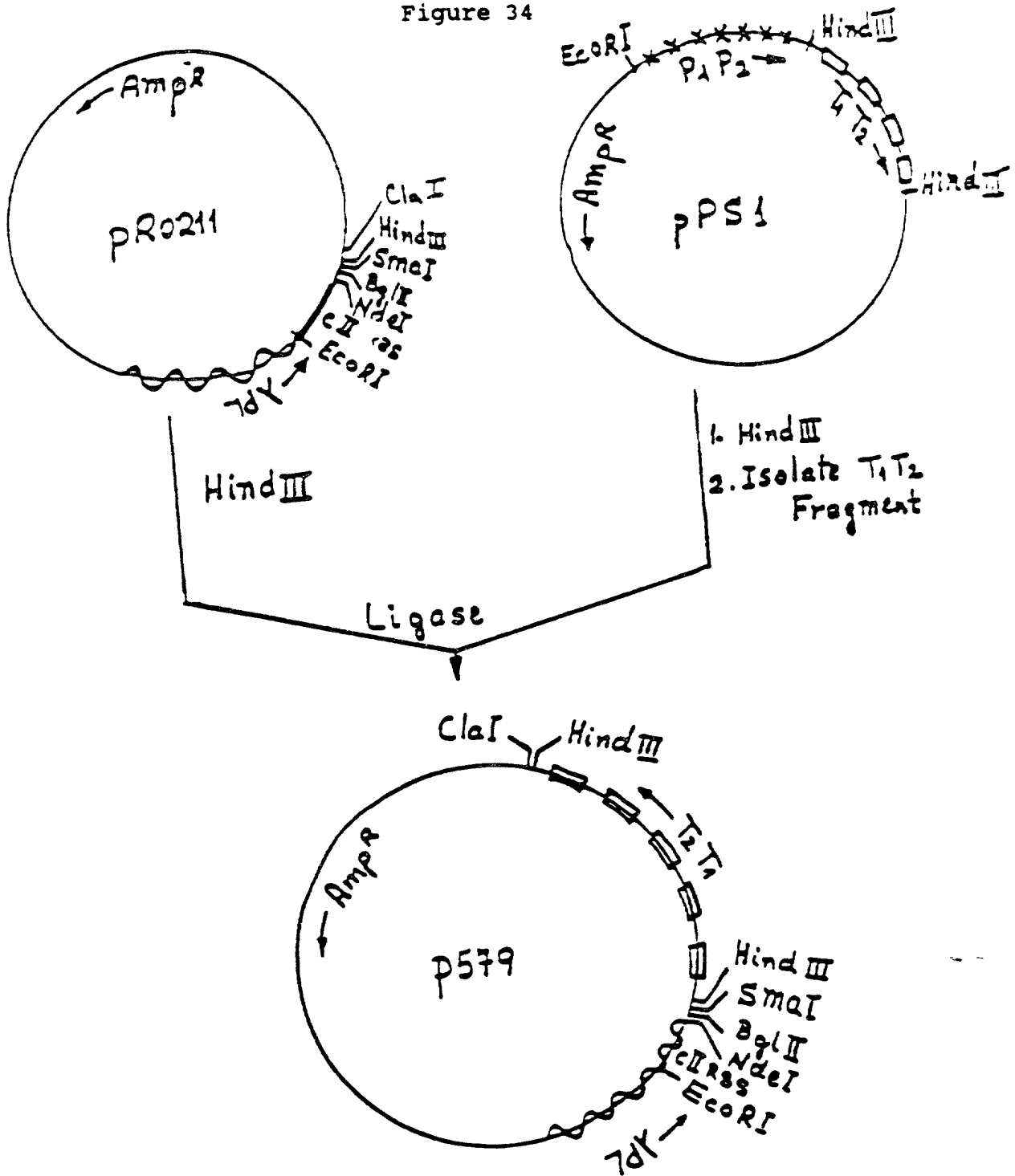
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Figure 33



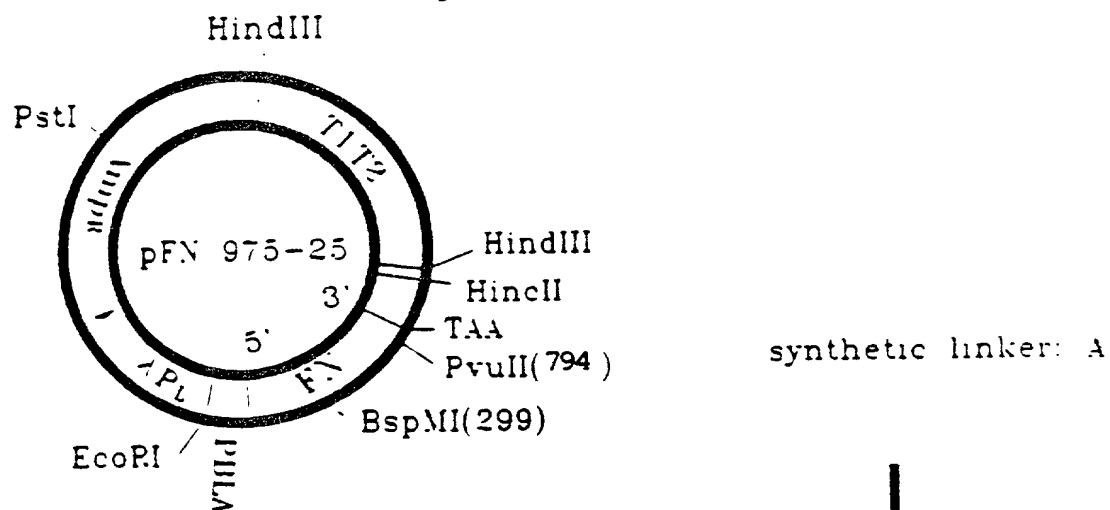
002270" T 2626460

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Figure 34



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Figure 36



1. digest with BspMI+HindIII
2. isolate large BspMI-HindIII fragment

T4 DNA ligase

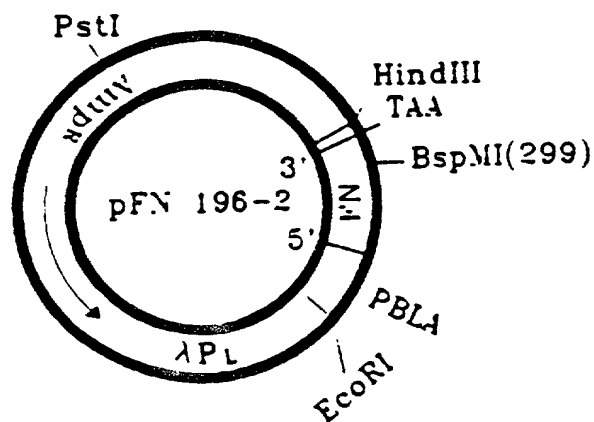
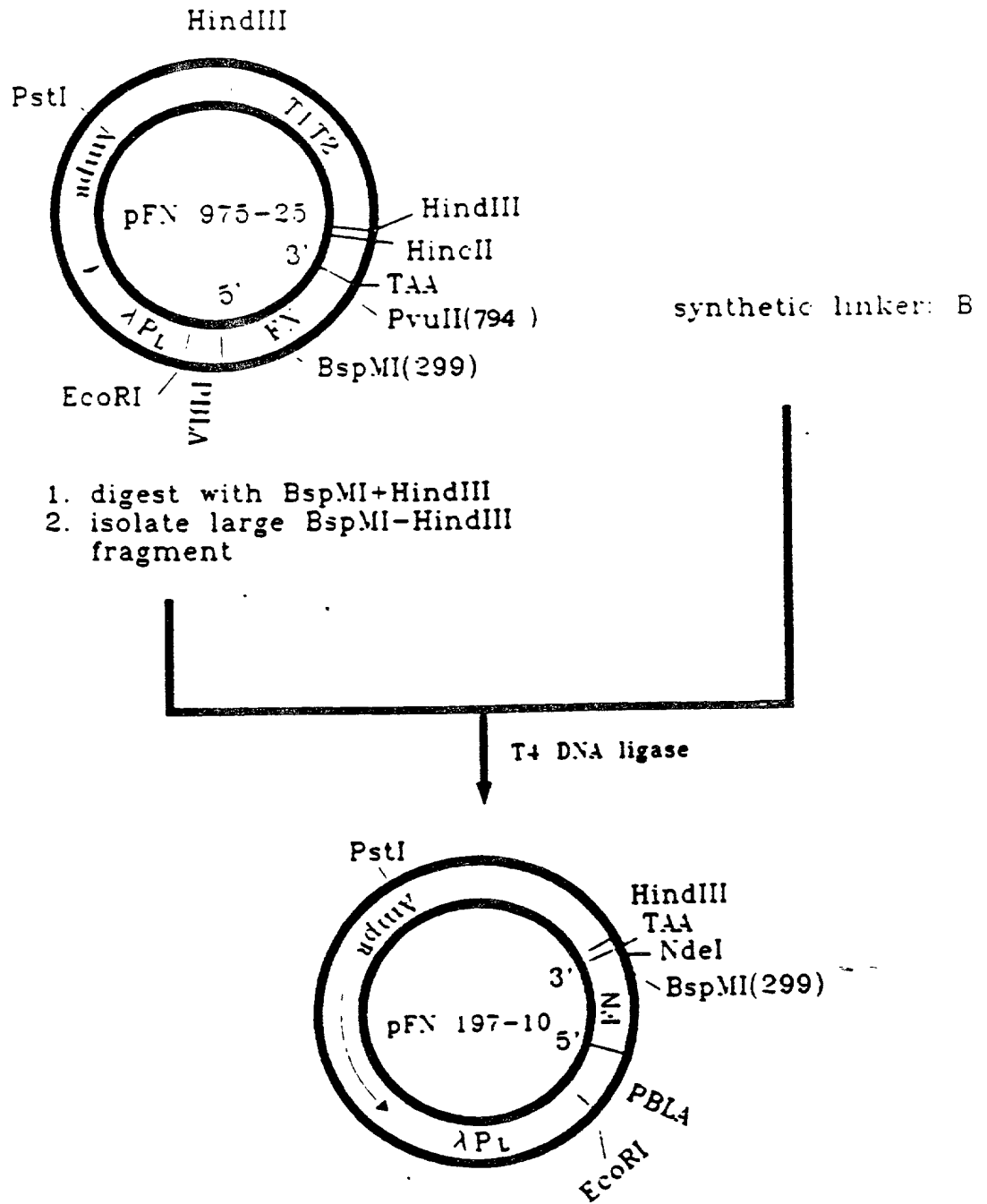


Figure 37



u/nb-5

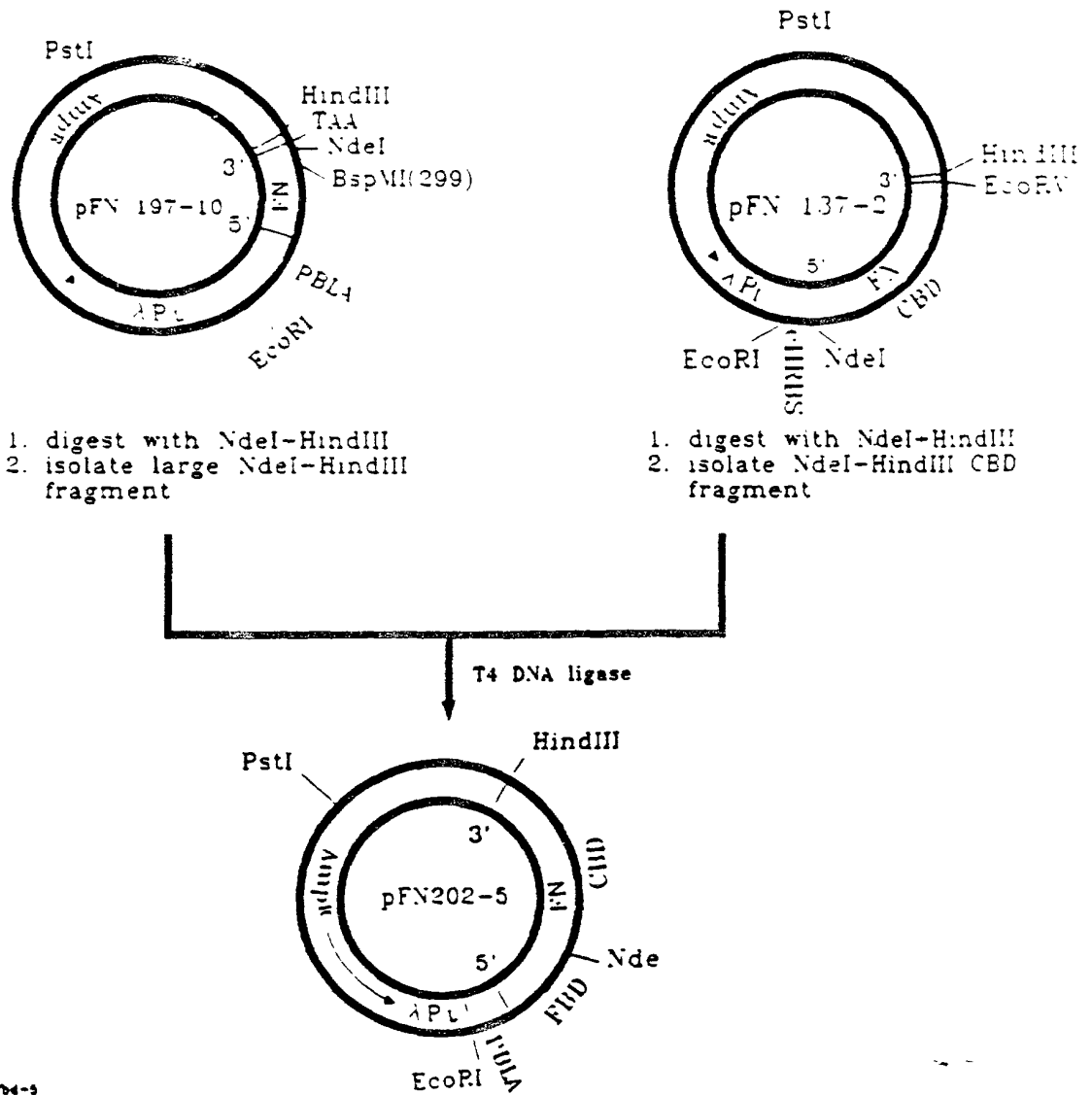
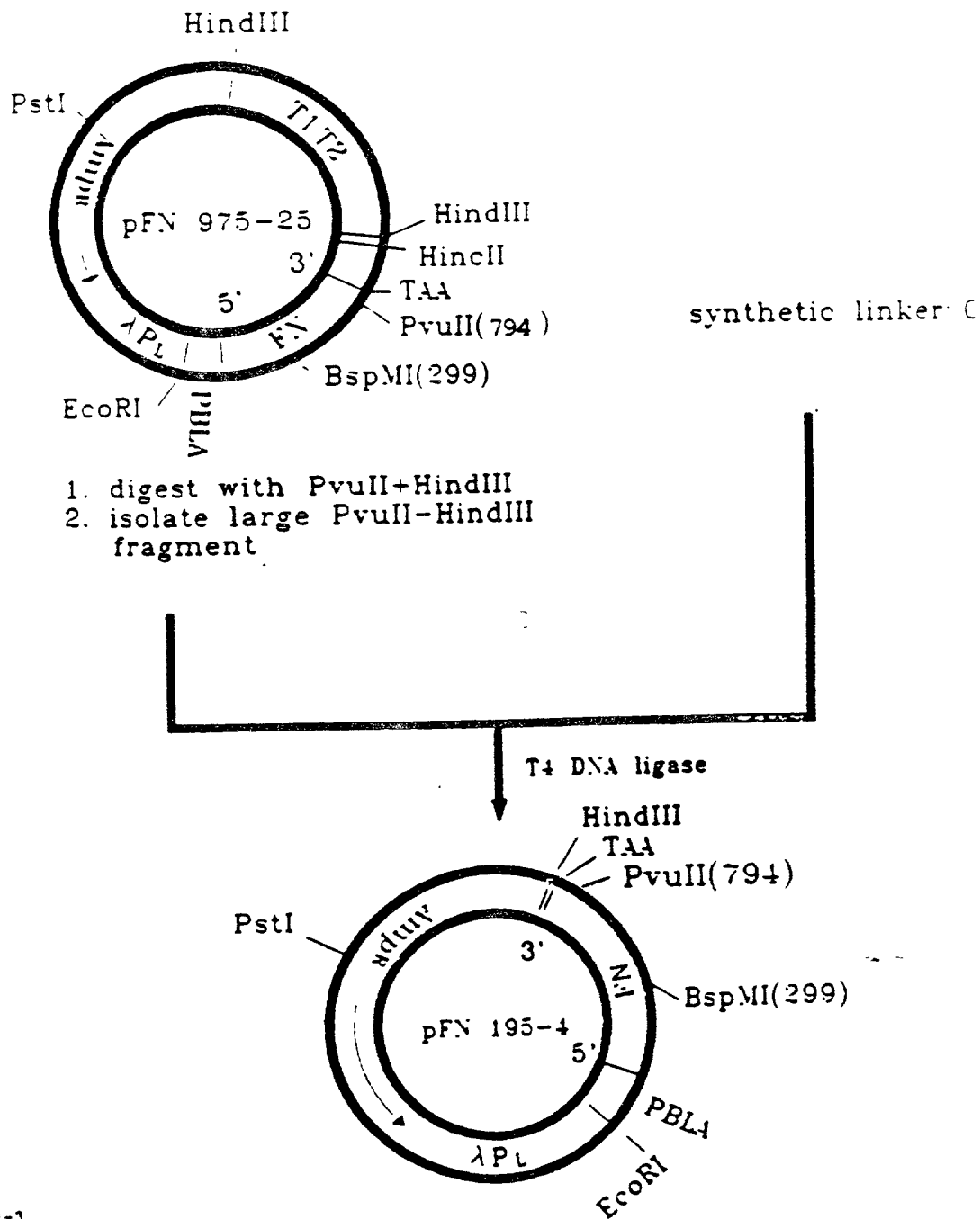
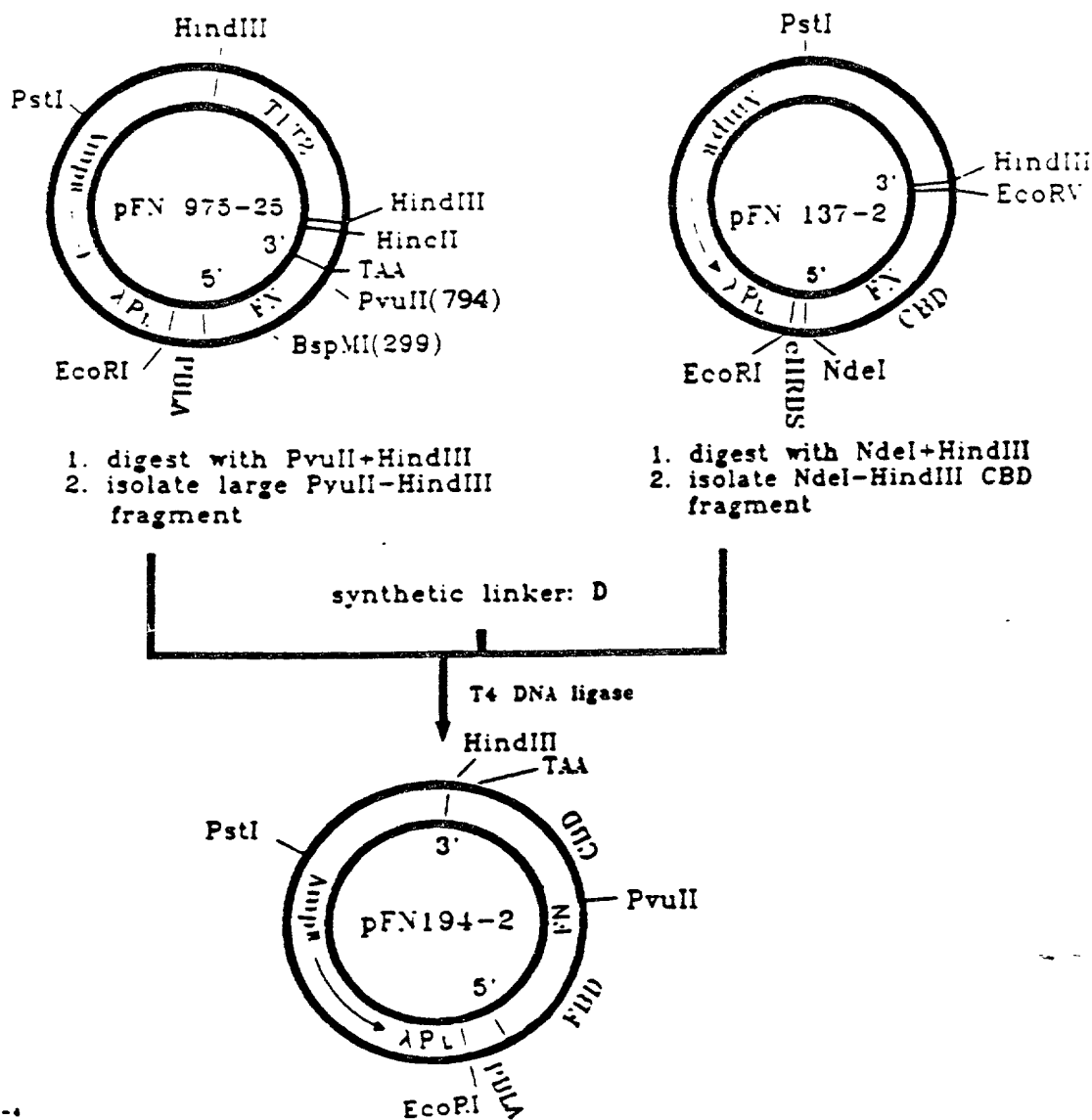


Figure 39



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Figure 40



A

```
5' GGGCTGGGCGAGGGAGAATAAGCTGTACCATCGCAAACCGCTAACAGCTGA 3'
3' ACCCGCTCCCTCTTATTCGACATGGTAGCGTTTGGCGATTGTCGACTTCGA 5'
```

B

```
5' GGGCTGGGCGAGGGAGAATAAGCTGTACCATCGCAAACCGCCATATGTAAA 3'
3' ACCCGCTCCCTCTTATTCGACATGGTAGCGTTTGGCGGTATACATTTTCGA 5'
```

C

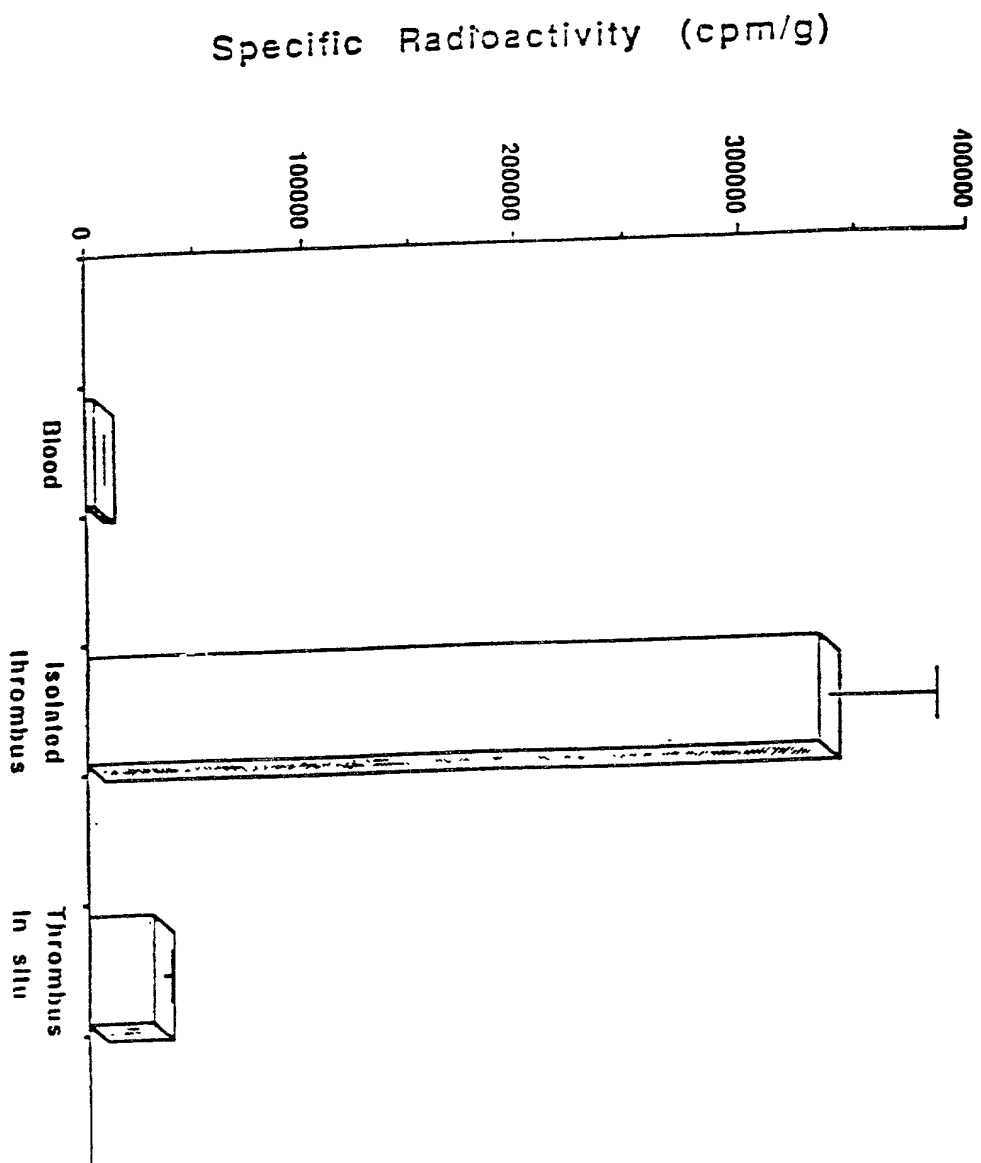
```
5' ATGGCCGTGGAGACAGCTAACAGCTGA 3'
3' TACCGGCACCTCTGTTCGATTGTTCGACTTCGA 5'
```

D

```
5' CTGTATACCAACC 3'
3' GACATATGGTTGGAT 5'
```

Uptake of Labeled 31KD rFBD by Blood Clots in the Rat Coil Model

Figure 42



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Figure 43

COMPARISON BETWEEN 12, 20 & 31KD FBD MOLECULES

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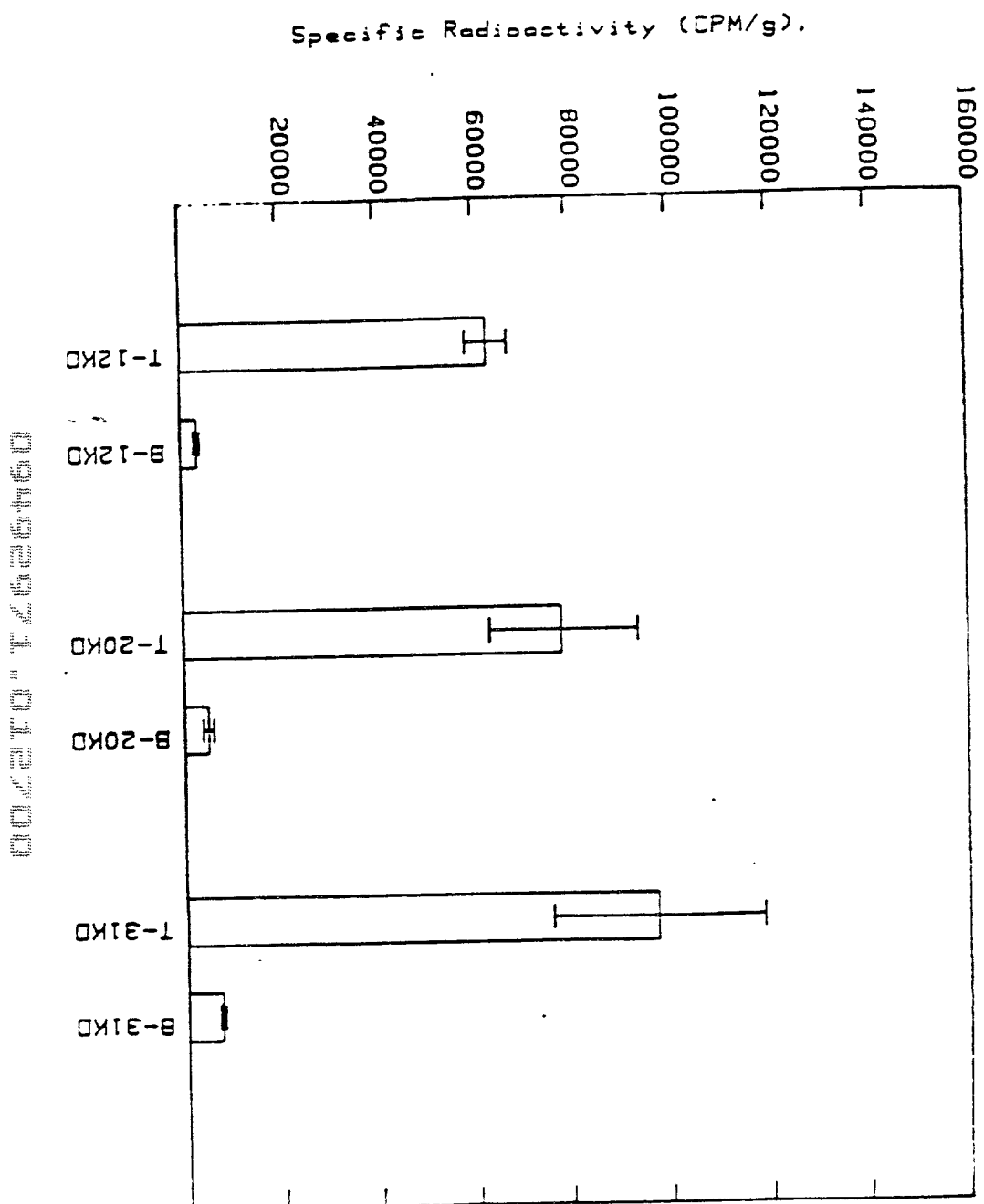
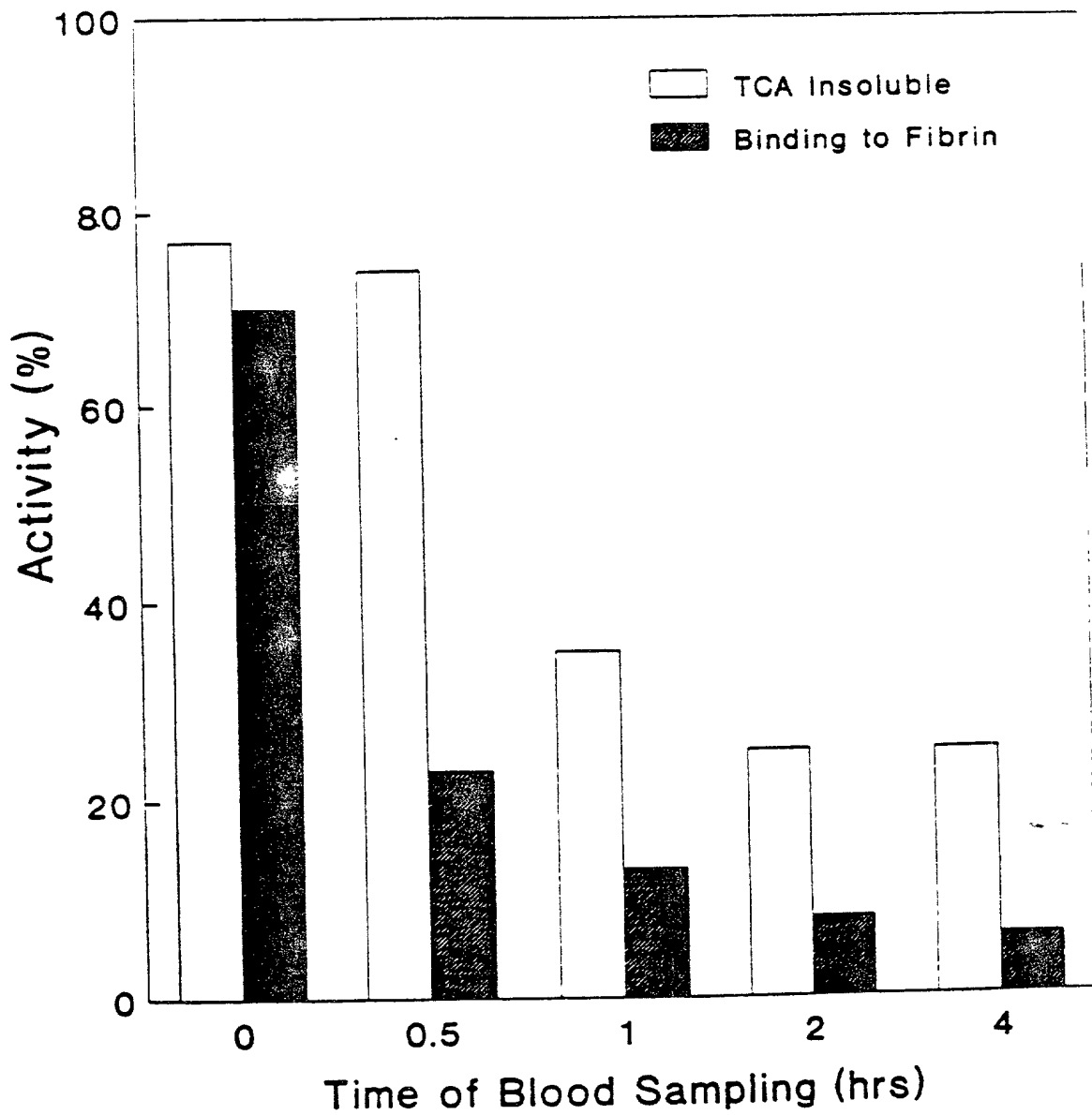


Figure 44

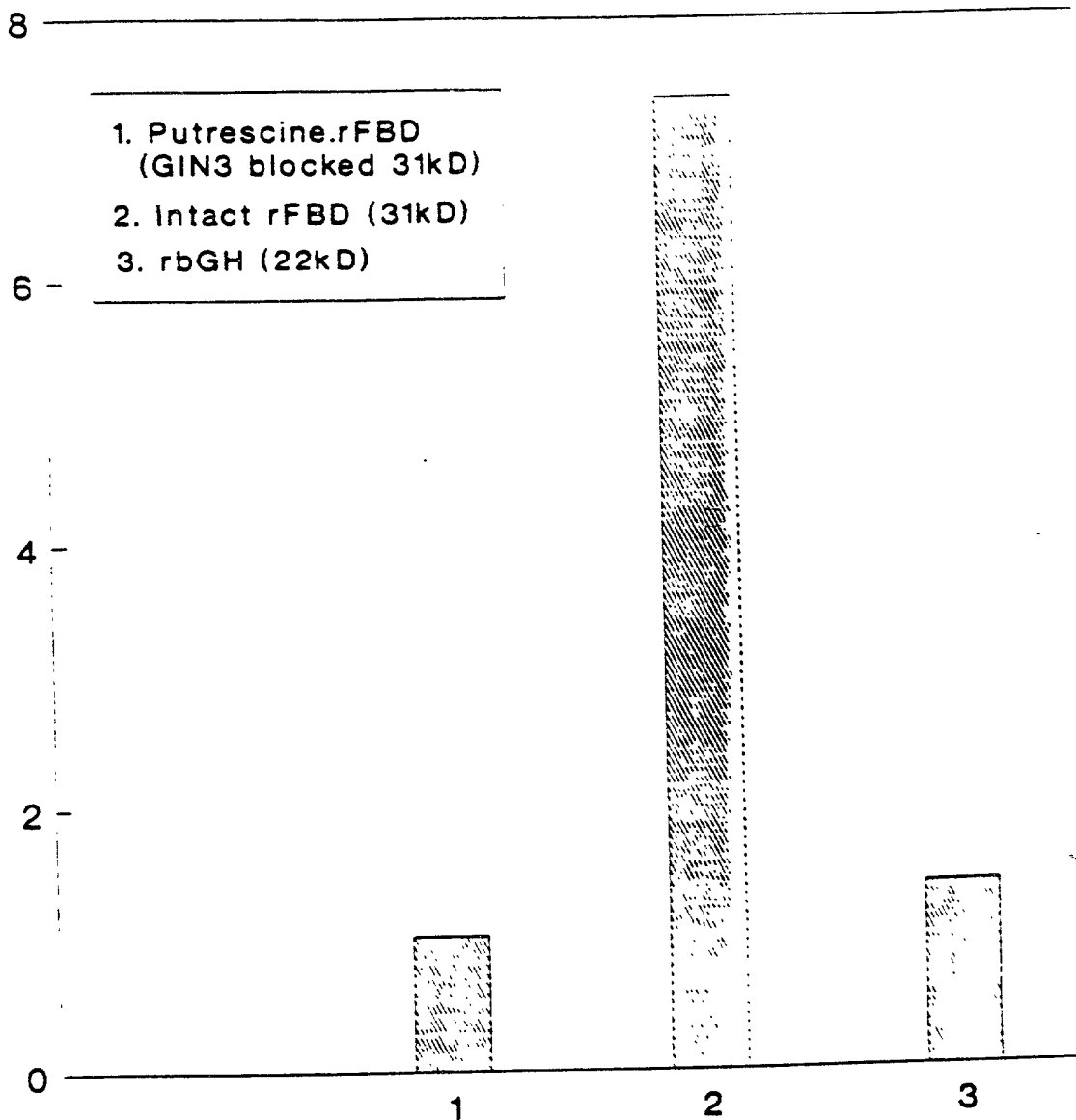
Metabolic Stability of rFBD in Rats;
Ex-vivo Binding to Fibrin vs. TCA
insolubility



ti-3/6/2

Figure 45

Specificity of binding to Fibrin;
Effect of T.G. on the binding of
rFBD vs. rbGH (Reaction II)



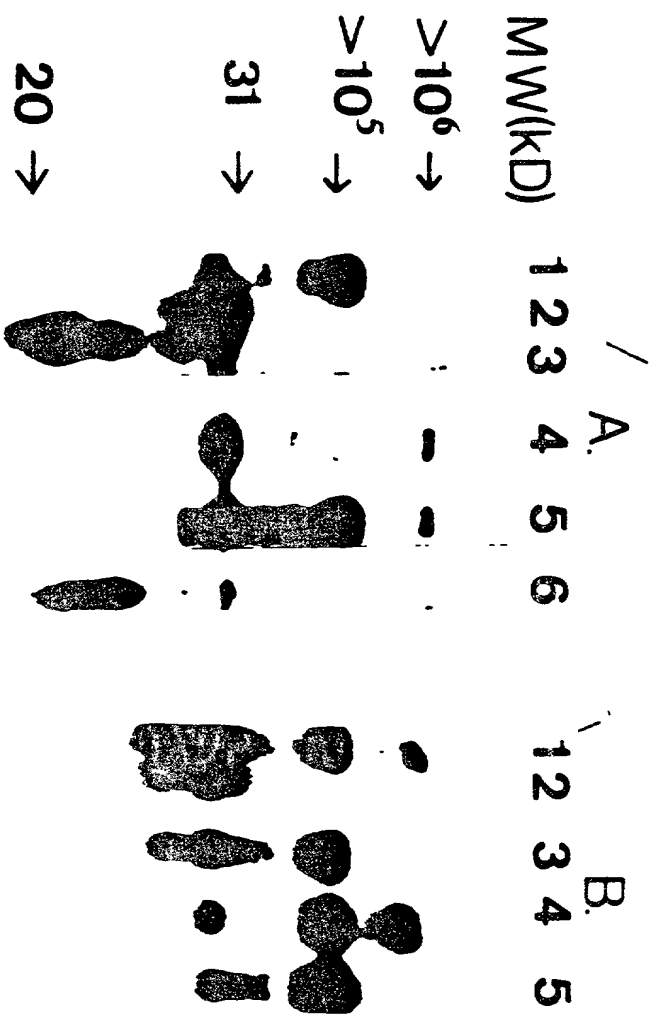
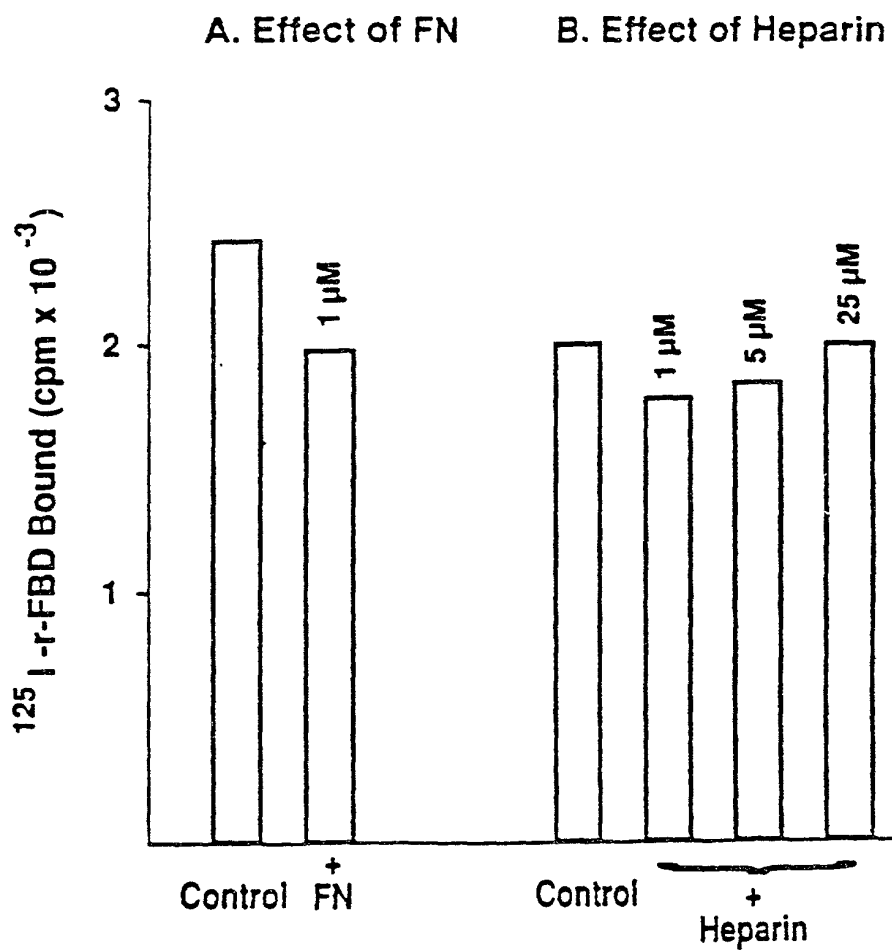


Figure 46

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Figure 47

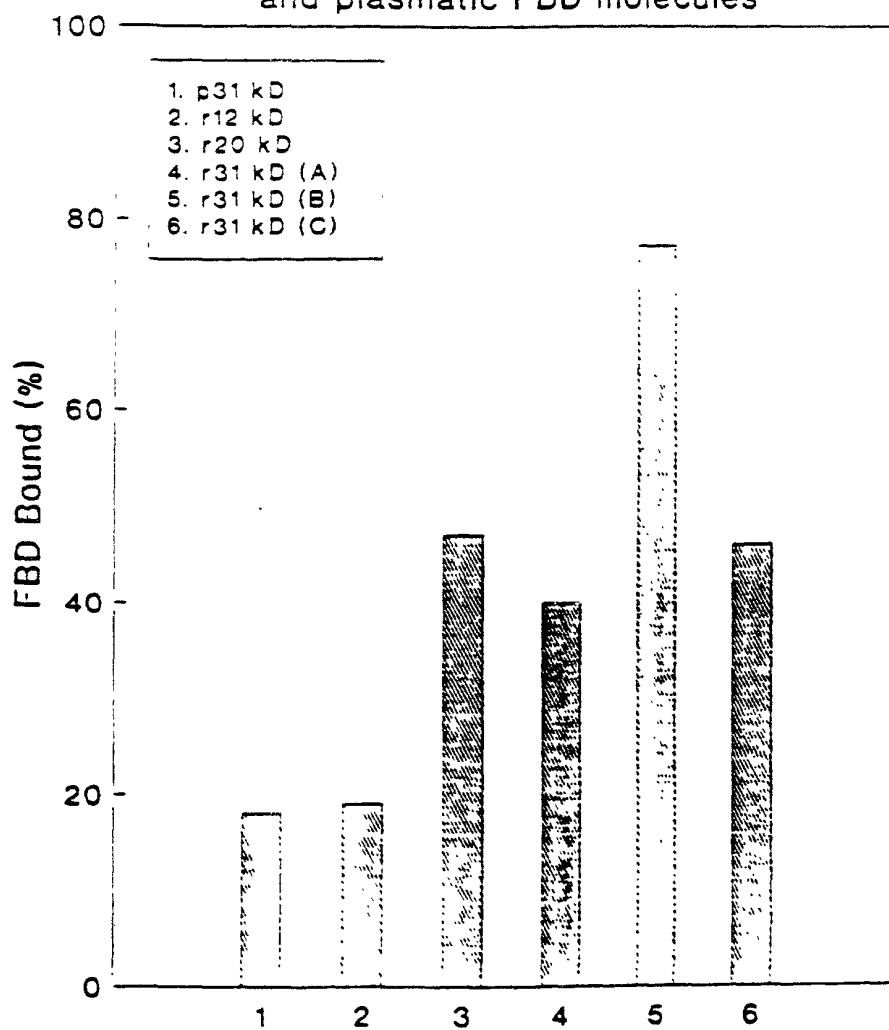
Binding of FBD to preformed clot (Reaction II);
Effect of FN and Heparin.



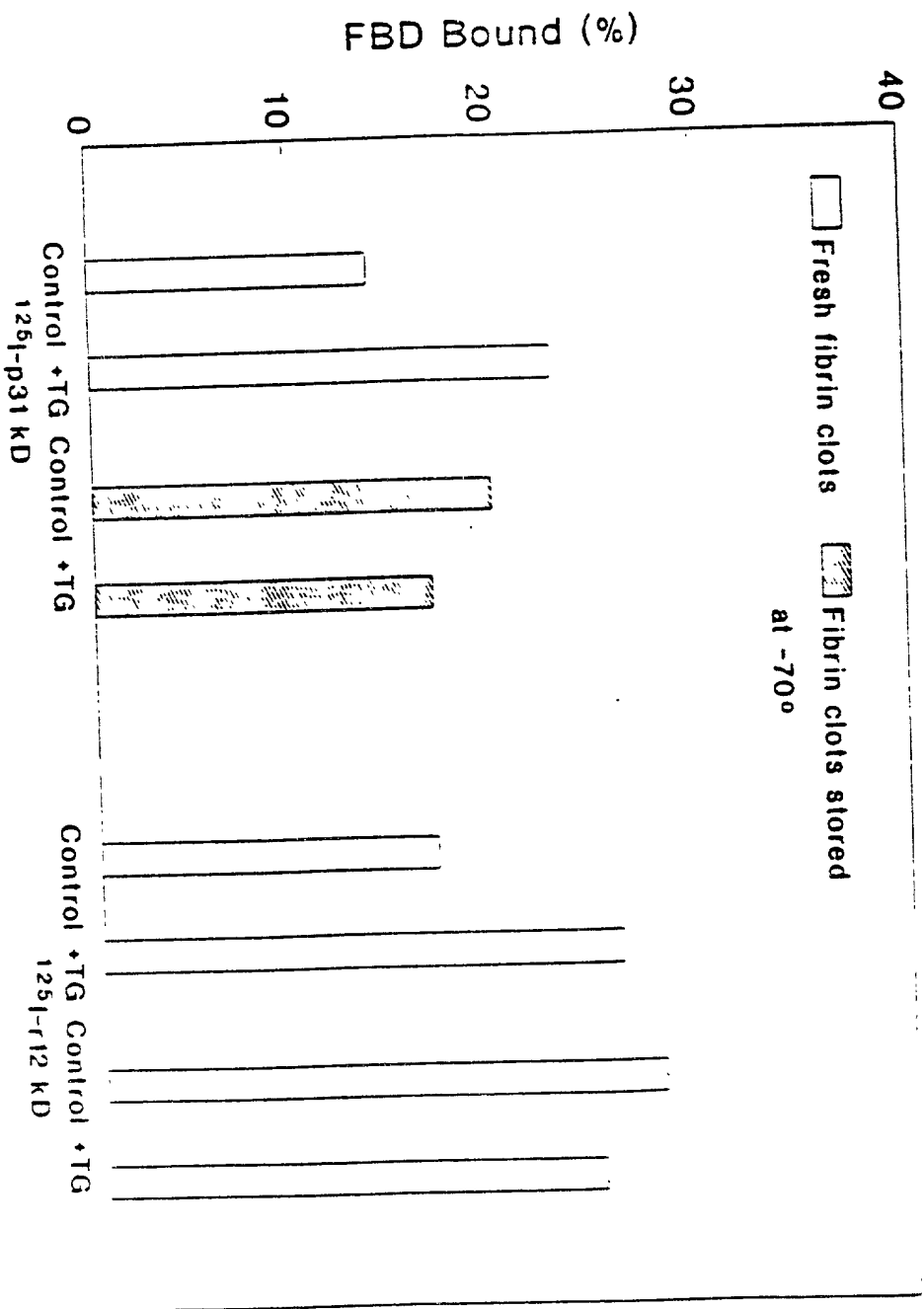
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Figure 48

Binding of FBD to Fibrin clot (reaction II);
comparison between various recombinant
and plasmatic FBD molecules



Binding of FBD to Fibrin clot (reaction II); Comparison between fresh and frozen Fibrin clots



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Figure 49

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Binding of FBD to Fibrin (Reaction II)

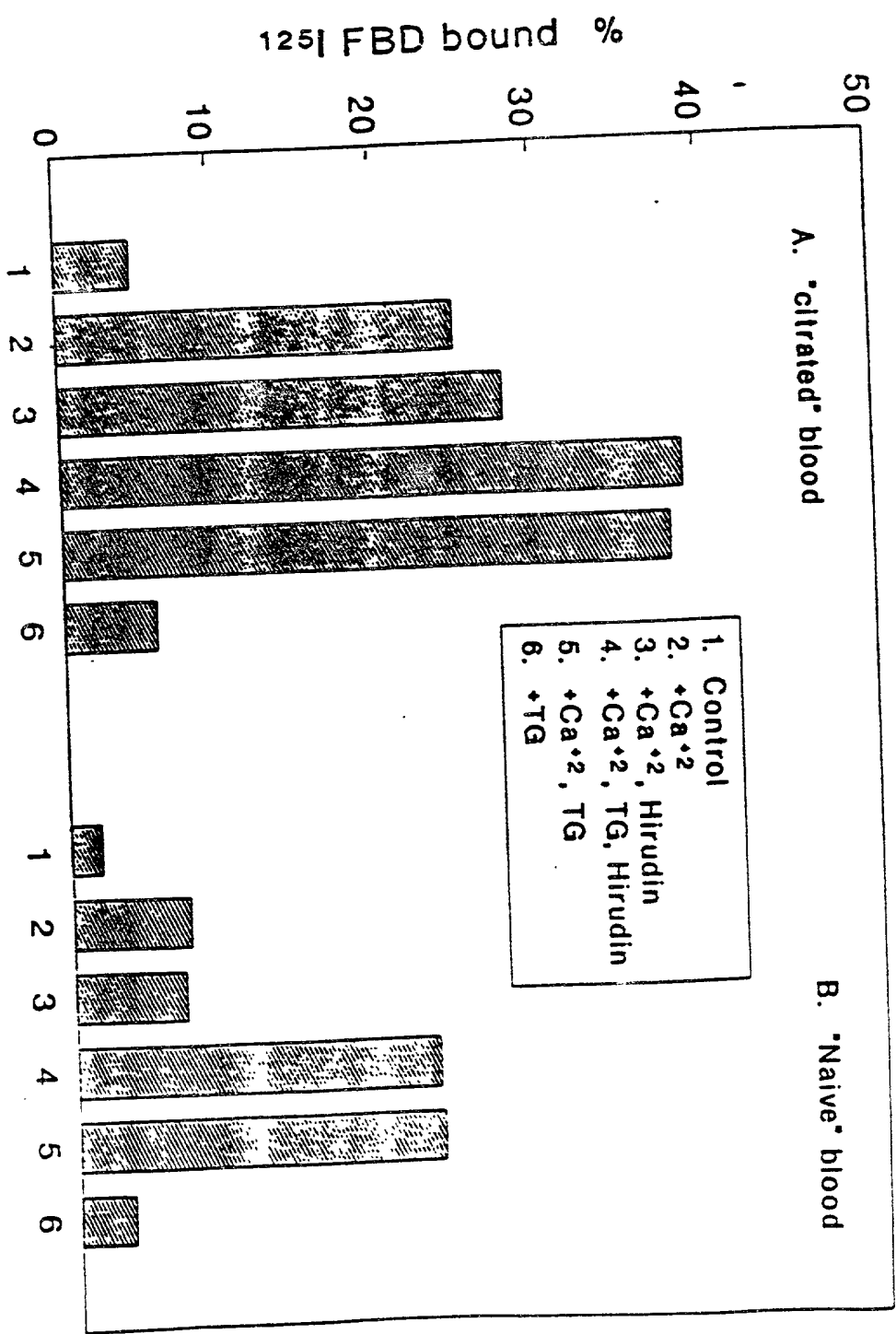
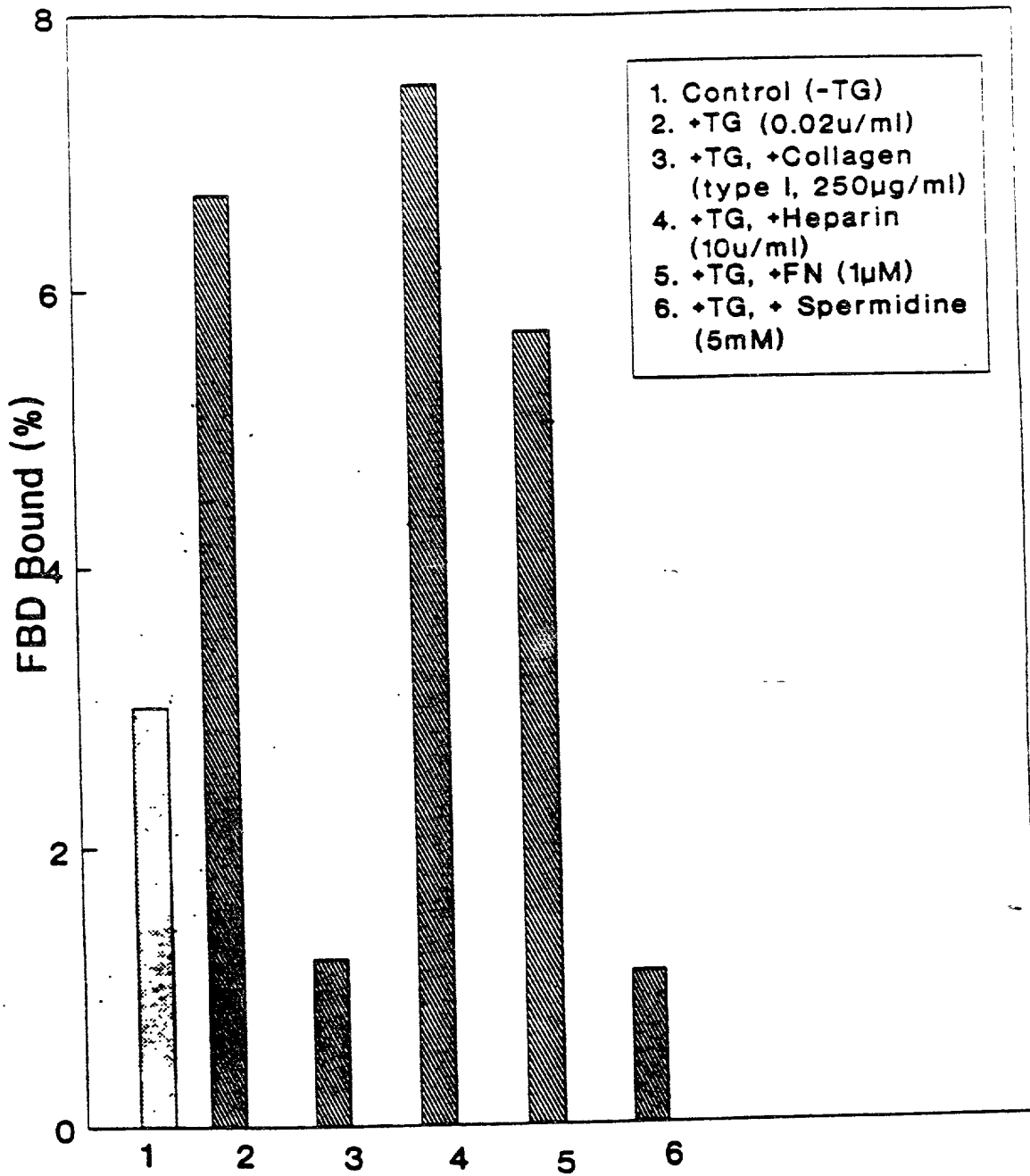


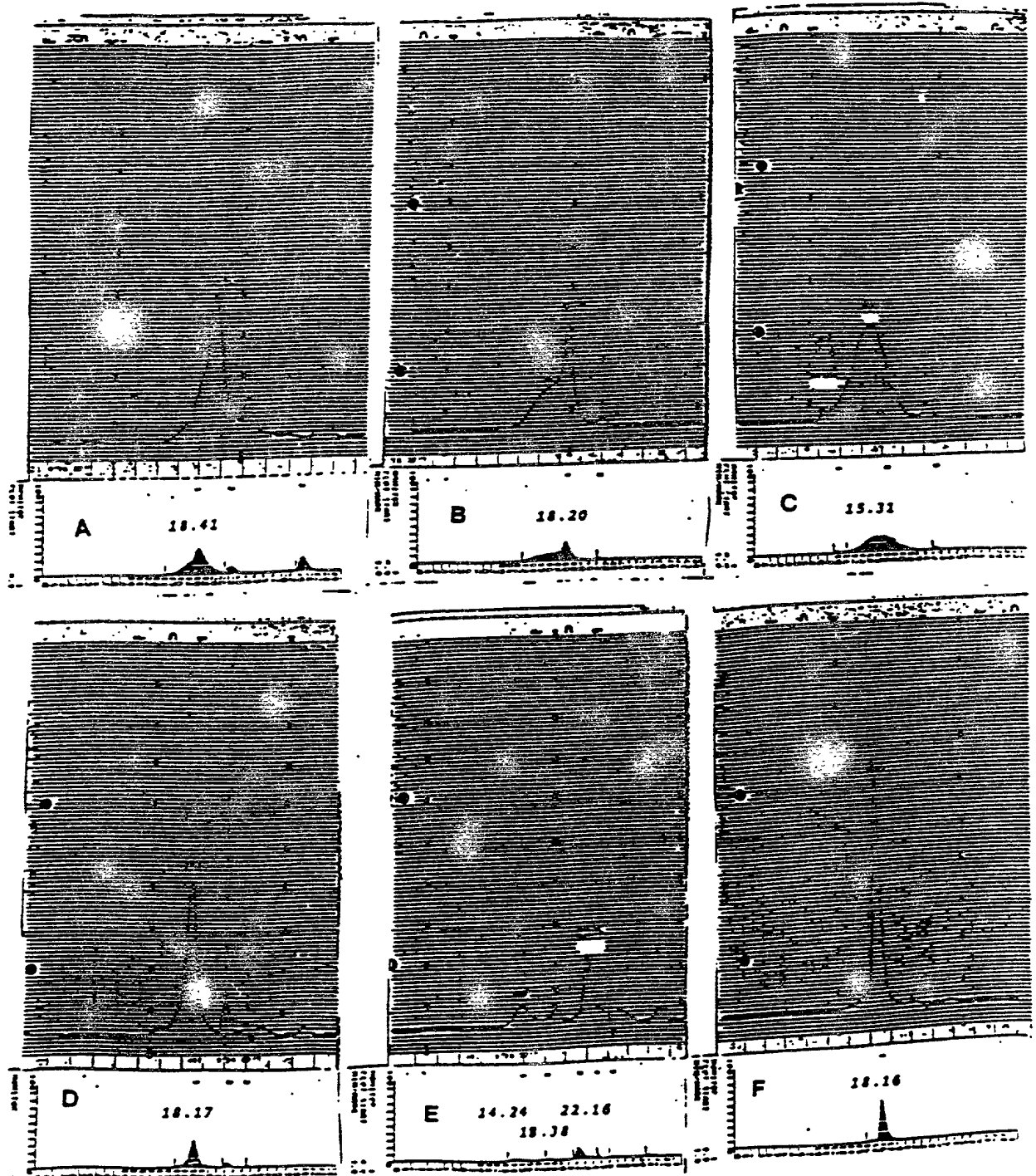
Figure 50
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Figure 51

r-FBD binding to ECM



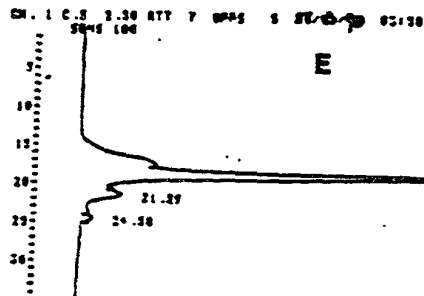
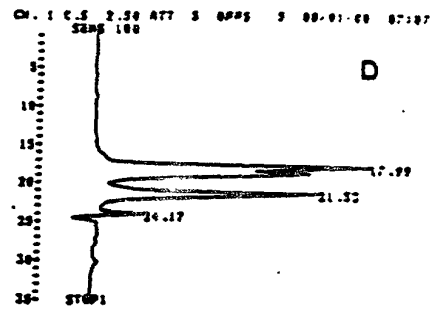
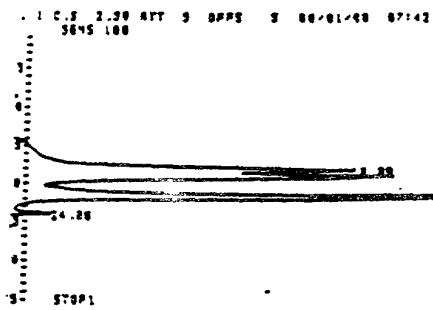
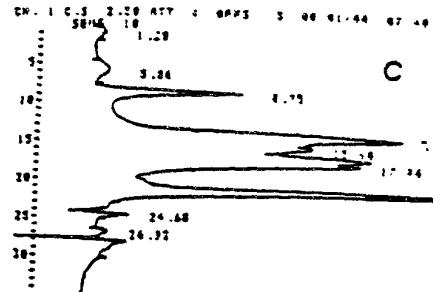
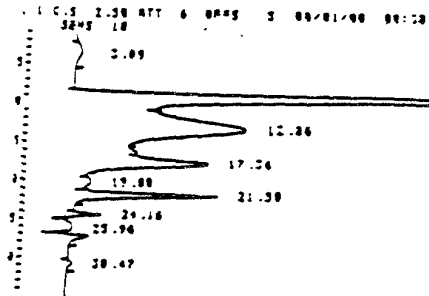
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Figure 52



00492971.012700

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Figure 53



004220" T 526460

Declaration and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

FIBRIN BINDING DOMAIN POLYPEPTIDES AND USES AND METHODS OF PRODUCING SAME

the specification of which
(check one)

_____ is attached hereto.

x was filed on May 21, 1990 as

Application Serial No. 526,397

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)		Filing Date	Priority Claimed	
Number	Country		Yes	No
<u>2,006,929-5</u>	<u>Canada</u>	<u>December 29, 1989</u>	<u>X</u>	
<u>US89/05875</u>	<u>PCT</u>	<u>December 29, 1989</u>		<u>X</u>
<u>092,925</u>	<u>Israel</u>	<u>December 29, 1989</u>		<u>X</u>

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sections 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
<u>345,952</u>	<u>April 28, 1989</u>	<u>Pending</u>
<u>291,951</u>	<u>December 29, 1988</u>	<u>Pending</u>

And I hereby appoint John P. White, Reg. No. 28,678; Thomas F. Moran, Reg. No. 16,579; Christopher C. Dunham, Reg. No. 22,031; Ivan S. Kavrukov, Reg. No. 25,161; Thomas G. Carulli, Reg. No. 30,616; Robert D. Katz, Reg. No. 30,141; Peter J. Phillips (Reg. No. 29,691; Antoinette F. Konski, Reg. No. P34,202; Catherine R. Roseman, Reg. No. P34,240; and Sarah B. Adriano, Reg. No. P34,470,

and each of them, all c/o Cooper & Dunham of 30 Rockefeller Plaza, New York, New York 10112 (Tel. 212 977-9550), my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to

John P. White, Esq. Reg. No. 28,678
Cooper & Dunham
30 Rockefeller Plaza
New York, N.Y. 10112
Tel. (212) 977-9550

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first joint inventor Tikva Vogel

Inventor's signature Tikva Vogel

Citizenship Israel Date of signature June 26th, 1991

Residence 4 Kosover Street, Rehovot, Israel

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00442971-012700

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Inventor's signature Avigdor Levanon
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Post Office Address Same as Above

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Inventor's signature M. Werber
Citizenship Israel Date June 27, 1990
Residence 36 Burla Street, Tel Aviv, Israel
Post Office Address Same as Above

Full name of joint inventor (if any) Rachel Guy
Inventor's signature Rachel Guy
Citizenship Israel Date 27/6/90
Residence 23 Miller Street, Rehovot, Israel
Post Office Address Same as Above

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Inventor's signature Amos Panet
Citizenship Israel Date 26.6.90
Residence 11 Harav Schrim Street, Jerusalem, Israel
Post Office Address Same as Above

002201-012700

FIGURE 1-1

S K R Q A Q Q M V Q P Q S P V
 1 5 10 15 49
 GAAG AGC AAG AGG CAG GCT CAG CAA ATG GTT CAG CCC CAG TCC CCG GTG

A V S Q S K P G C Y D N G K H Y
 20 25 30 97
 GCT GTC AGT CAA AGC AAG CCC GGT TGT TAT GAC AAT GGA AAA CAC TAT

Q I N Q Q W E R T Y L G N V L V
 35 40 45 145 1/00
 CAG ATA AAT CAA CAG TGG GAG CCG ACC TAC CTA GGT AAT GTG TTG GTT

C T C Y G G S R G F N C E S K P
 50 55 60 193
 TGT ACT TGT TAT GGA GGA AGC CGA GGT TTT AAC TGC GAA AGT AAA CCT

E A E E T C F D K Y T G N T Y R
 65 70 75 241
 GAA GCT GAA GAG ACT TGC TTT GAC AAG TAC ACT GGG AAC ACT TAC CGA

V G D T Y E R P K D S M I W D C
 80 85 90 95 289
 GTG GGT GAC ACT TAT GAG CGT CCT AAA GAC TCC ATG ATC TGG GAC TGT

FIGURE 1-2

T C I G A G R G R I S C T I A N
ACC TGC ATC GGG GCT GGG CGA GGG AGA ATA AGC TGT ACC ATC GCA AAC 337
100 105 110

R C H E G G Q S Y K I G D T W R
CGC TGC CAT GAA GGG GGT CAG TCC TAC AAG ATT GGT GAC ACC TGG AGG 385
115 120 125

R P H E T G G Y M L E C V C L G
AGA CCA CAT GAG ACT GGT GGT TAC ATG TTA GAG TGT GTG TGT CTT GGT 433
130 135 140

N G K G E W T C K P I A E K C F
AAT GGA AAA GGA GAA TGG ACC TGC AAG CCC ATA GCT GAG AAG TGT TTT 481
145 150 155

D H A A G T S Y V V G E T W E K
GAT CAT GCT GCT GGG ACT CCC TAT GTG GTC GGA GAA ACG TGG GAG AAG 529
160 165 170 175

FIGURE 1-3

P Y Q G W M M V D C T C L G E G
 CCC TAC CAA GGC TGG ATG ATG GTA GAT TGT ACT TGC CTG GGA GAA GGC 577
 180 185 190

S G R I T C T S R N R C N D Q D
 AGC GGA CGC ATC ACT TGC ACT TCT AGA AAT AGA TGC AAC GAT CAG GAC 625
 195 200 205

T R T S Y R I G D T W S K K D N
 ACA AGG ACA TCC TAT AGA ATT TGA GAC ACC TGG AGC AAG AAG GAT AAT 673
 210 215 220 3/8

R G N L L Q C I C T G N G R G E
 CGA GGA AAC CTG CTC CAG TGC ATC TGC ACA GGC AAC GGC CGA GGA GAG 721
 225 230 235

W K C E R H T S V Q T T S S G S
 TGG AAG TGT GAG AGG CAC ACC TCT GTG CAG ACC ACA TCG AGC GGA TCT 769
 240 245 250 255

G P F T D V R A A V Y Q P Q P H
 GGC CCC TTC ACC GAT GTT CGT GCA GCT GTT TAC CAA CCG CAG CCT CAC 817
 260 265 270

4/86

FIGURE 1-5

Q D G H L W C S T T S N Y E Q D
CAG GAC GGA CAT CTT TGG TGC AGC ACA ACT TCG AAT TAT GAG CAG GAC 1105
355 360 365

Q K Y S F C T D H T V L V Q T Q
CAG AAA TAC TCT TTC TGC ACA GAC CAC ACT GTT TTG GTT CAG ACT CAA 1153
370 375 380

G G N S N G A L C H F P F L Y N
GGA GGA AAT TCC AAT GGT GCC TTG TGC CAC TTC CCC TTC CTA TAC AAC 1201
385 390 395

N H N Y T D C T S E G R R D N M
AAC CAC AAT TAC ACT GAT TGC ACT TCT GAG GGC AGA AGA GAC AAC ATG 1249
400 405 410 415

K W C G T T Q N Y D A D Q K F G
AAG TGG TGT GGG ACC ACA CAG AAC TAT GAT GCC GAC CAG AAG TTT GGG 1297
420 425 430

F C P M A A H E E I C T T N E G
TTC TGC CCC ATG GCT GCC CAC GAG GAA ATC TGC ACA ACC AAT GAA GGG 1345
435 440 445

FIGURE 1-6

V	M	Y	R	I	G	D	Q	W	D	K	Q	H	D	M	G	1393
GTC	ATG	TAC	CGC	ATT	GGA	GAT	CAG	TGG	GAT	AAG	CAG	CAT	GAC	ATG	GGT	
450							455					460				
H	M	M	R	C	T	C	V	G	N	G	R	G	E	W	T	1441
CAC	ATG	ATG	AGG	TGC	ACG	TGT	GTT	GGG	AAT	GGT	CGT	GGG	GAA	TGG	ACA	
465						470					475					
C	I	A	Y	S	Q	L	R	D	Q	C	I	V	D	D	I	1489
TGC	ATT	GCC	TAC	TCG	CAA	CTT	CGA	GAT	CAG	TGC	ATT	GTT	GAT	GAC	ATC	
480					485					490					495	
T	Y	N	V	N	D	T	F	H	K	R	H	E	E	G	H	1537
ACT	TAC	AAT	GTG	AAC	GAC	ACA	TTC	CAC	AAG	CGT	CAT	GAA	GAG	GGG	CAC	
500									505					510		
M	L	N	C	T	C	F	G	Q	G	R	G	R	W	K	C	1585
ATG	CTG	AAC	TGT	ACA	TGC	TTC	GGT	CAG	GGT	CGG	GGC	AGG	TGG	AAG	TGT	
515								520					525			

FIGURE 1-7

D P V D Q C Q D S E T G T F Y Q
GAT CCC GTC GAC CAA TGC CAG GAT TCA GAG ACT GGG ACG TTT TAT CAA
530 535 540 1633

I G D S W E K Y V H G V R Y Q C
ATT GGA GAT TCA TGG GAG AAG TAT GTG CAT GGT GTC AGA TAC CAG TGC
545 550 555 1681

Y C Y G R G I G E W H C Q P L Q
TAC TGC TAT GGC CGT GGC ATT GGG GAG TGG CAT TGC CAA CCT TTA CAG
560 565 570 1729

T Y P S S S G P V E V F I T E T
ACC TAT CCA AGC TCA AGT GGT CCT GTC GAA GTA TTT ATC ACT GAG ACT
580 585 590 1777

P S Q P N S H P I Q W N A P Q P
CCG AGT CAG CCC AAC TCC CAC CCC ATC CAG TGG AAT GCA CCA CAG CCA
595 600 605 1825

S H I S K Y I L R W R P K N S V
TCT CAC ATT TCC AAG TAC ATT CTC AGG TGG AGA CCT AAA AAT TCT GTA
610 615 620 1873

FIGURE 1-8

G R W K E A T I P G H L N S Y T
 GGC CGT TGG AAG GAA GCT ACC ATA CCA GGC CAC TTA AAC TCC TAC ACC 1921
 625 630 635

I K G L K P G V V Y E G Q L I S
 ATC AAA GGC CTG AAG CCT GGT GTG GTA TAC GAG GGC CAG CTC ATC AGC 1969
 640 645 650 655

8/85

I Q Q Y G H Q E V T T R F D F T T
 ATC CAG CAG TAC GGC CAC CAA GAA GTG ACT CGC TTT GAC TTC ACC ACC 2017
 660 665 670

T S T S T P V T S N T V T G E T
 ACC AGC ACC AGC ACA CCT GTG ACC AGC AAC ACC GTG ACA GGA GAG ACC 2065
 675 680 685

T P F S P L V A T S E S V T E I
 ACT CCC TTT TCT CCT CTT GTG GCC ACT TCT GAA TCT GTG ACC GAA ATC 2113
 690 695 700

FIGURE 1-9

T A S S F V V S W V S A S D T V
 ACA GCC AGT AGC TTT GTG GTC TCC TGG GTC TCA GCT TCC GAC ACC GTG 2161
 705 710 715

S G F R V E Y E L S E E G D E P
 TCG GGA TTC CGG GTG GAA TAT GAG CTG AGT GAG GAG GGA GAT GAG CCA 2209
 720 725 730 735

Q Y L D L P S T A T S V N I P D
 CAG TAC CTG GAT CTT CCA AGC ACA GCC ACT TCT GTG AAC ATC CCT GAC 2257
 740 745 750

L L P G R K Y I V N V Y Q I S E
 CTG CTT CCT GGC CGA AAA TAC ATT GTA AAT GTC TAT CAG ATA TCT GAG 2305
 755 760 765

D G E Q S L I L S T S Q T T A P
 GAT GGG GAG CAG AGT TTG ATC CTG TCT ACT TCA CAA ACA ACA GCG CCT 2353
 770 775 780

D A P P D P T V D Q V D D T S I
 GAT GCC CCT CCT GAC CCG ACT GTG GAC CAA GTT GAT GAC ACC TCA ATT 2401
 785 790 795

FIGURE 1-10

V V R W S R P Q A P I T G Y R I
 GTT GTT CGC TGG AGC AGA CCC CAG GCT CCC ATC ACA GGG TAC AGA ATA
 800 805 810 815 2449

V Y S P S V E G S S T E L N L P
 GTC TAT TCG CCA TCA GTA GAA GGT AGC AGC ACA GAA CTC AAC CTT CCT
 820 825 830 2497

E T A N S V T L S D L Q P G V Q
 GAA ACT GCA AAC TCC GTC ACC CTC AGT GAC TTG CAA CCT GGT GTT CAG
 835 840 845 2545
 10/86

Y N I T I Y A V E E N Q E S T P
 TAT AAC ATC ACT ATC TAT GCT GTG GAA GAA AAT CAA GAA AGT ACA CCT
 850 855 860 2593

V V I Q Q E T T G T P R S D T V
 GTT GTC ATT CAA CAA GAA ACC ACT GGC ACC CCA CGC TCA GAT ACA GTG
 865 870 875 2641

FIGURE 1-11

P S P R D L Q F V E V T D V K V
 CCC TCT CCC AGG GAC CTG CAG TTT GTG GAA GTG ACA GAC GTG AAG GTC
 880 885 890 895 2689

T I M W T P P E S A V T G Y R V
 ACC ATC ATG TGG ACA CCG CCT GAG AGT GCA GTG ACC GGC TAC CGT GTG
 900 905 910 2737

D V I P V N L P G E H G Q R L P
 GAT GTG ATC CCC GTC AAC CTG CCT GGC GAG CAC GGG CAG AGG CTG CCC
 915 920 925 2785

11/85

I S R N T F A E V T G L S P G V
 ATC AGC AGG AAC ACC TTT GCA GAA GTC ACC GGG CTG TCC CCT GGG GTC
 930 935 940 2833

T Y Y F K V F A V S H G R E S K
 ACC TAT TAC TTC AAA GTC TTT GCA GTG AGC CAT GGG AGG GAG AGC AAG
 945 950 955 2881

P L T A Q Q T T K L D A P T N L
 CCT CTG ACT GCT CAA CAG ACA ACC AAA CTG GAT GCT CCC ACT AAC CTC
 960 965 970 975 2929

FIGURE 1-12

Q F V N E T D S T V L V R W T P
CAG TTT GTC AAT GAA ACT GAT TCT ACT GTC CTG GTG AGA TGG ACT CCA 2977
980 985 990

P R A Q I T G Y R L T V G L T R
CCT CGG GCC CAG ATA ACA GGA TAC CGA CTG ACC GTG GGC CTT ACC CGA 3025
995 1000 1005

12/86

R G Q P R Q Y N V G P S V S K Y
AGA GGC CAG CCC AGG CAG TAC AAT GTG GGT CCC TCT GTC TTC AAG TAC 3073
1010 1015 1020

P L R N L Q P A S E Y T V S L V
CCC CTG AGG AAT CTG CAG CCT GCA TCT GAG TAC ACC GTA TCC CTC GTG 3121
1025 1030 1035

A I K G N Q E S P K A T G V F T
GCC ATA AAG GGC AAC CAA GAG AGC CCC AAA GCC ACT GGA GTC TTT ACC 3169
1040 1045 1050 1055

FIGURE 1-13

T L Q P G S S I P P Y N T E V T
 ACA CTG CAG CCT GGG AGC TCT ATT CCA CCT TAC AAC ACC GAG GTG ACT 3217
 1060 1065 1070

E T T I V I T W T P A P R I G F
 GAG ACC ACC ATC GTG ATC ACA TGG ACG CCT GCT CCA AGA ATT GGT TTT 3265
 1075 1080 1085

K L G V R P S Q G G E A P R E V
 AAG CTG GGT GTA CGA CCA AGC CAG GGA GGA GAG GCA CCA CGA GAA GTG 3313
 1090 1095 1100

T S D S G S I V V S G L T P G V
 ACT TCA GAC TCA GGA AGC ATC GTT GTG TCC GGC TTG ACT CCA GGA GTA 3361
 1105 1110 1115

E Y V Y Y T I Q V L R D G Q E R D
 GAA TAC GTC TAC ACC ATC CAA GTC CTG AGA GAT GGA CAG GAA AGA GAT 3409
 1120 1125 1130 1135

A P I V N K V V T P L S P P T N
 GCG CCA ATT GTA AAC AAA GTG GTG ACA CCA TTG TCT CCA CCA ACA AAC 3457
 1140 1145 1150

FIGURE 1-14

L H L E A N P D T G V L T V S W
 TTG CAT CTG GAG GCA AAC CCT GAC ACT GGA GTG CTC ACA GTC TCC TGG
 1155 1160 1165 3505

E R S T T P D I T G Y R I T T
 GAG AGG AGC ACC ACC CCA GAC ATT ACT GGT TAT AGA ATT ACC ACA ACC
 1170 1175 1180 3553

P T N G Q Q G N S L E E V V H A
 CCT ACA AAC GGC CAG CAG GGA AAT TCT TTG GAA GAA GTG GTC CAT GCT
 1185 1190 1195 3601

D Q S S C T F D N L S P G L E Y
 GAT CAG AGC TCC TGC ACT TTT GAT AAC CTG AGT CCC GGC CTG GAG TAC
 1200 1205 1210 1215 3649

N V S V Y T V K D D K E S V P I
 AAT GTC AGT GTT TAC ACT GTC AAG GAT GAC AAG GAA AGT GTC CCT ATC
 1220 1225 1230 3697

FIGURE 1-15

S D T I I P A V P P P T D L R F
TCT GAT ACC ATC ATC CCA GCT GTT CCT CCT CCC ACT GAC CTG CGA TTC 3745
1235 1240 1245

T N I G P D T M R V T W A P P P
ACC AAC ATT GGT CCA GAC ACC ATG CGT GTC ACC TGG GCT CCA CCC CCA 3793
1250 1255 1260

S I D L T N F L V R Y S P V K N
TCC ATT GAT TTA ACC AAC TTC CTG GTG CGT TAC TCA CCT GTG AAA AAT 3841
1265 1270 1275 15/80

E E D V A E L S I S P S D N A V
GAG GAA GAT GTT GCA GAG TTG TCA ATT TCT CCT TCA GAC AAT GCA GTG 3889
1280 1285 1290 1295

V L T N L L P G T E Y V V S V S
GTC TTA ACA AAT CTC CTG CCT GGT ACA GAA TAT GTA GTG AGT GTC TCC 3937
1300 1305 1310

S V Y E Q H E S T P L R G R Q K
AGT GTC TAC GAA CAA CAT GAG AGC ACA CCT CTT AGA GGA AGA CAG AAA 3985
1315 1320 1325

FIGURE 1-16

T G L D S P T G I D F S D I T A
ACA GGT CTT GAT TCC CCA ACT GGC ATT GAC TTT TCT GAT ATT ACT GCC 4033
1330 1335 1340

N S F T V H W I A P R A T I T G
AAC TCT TTT ACT GTG CAC TGG ATT GCT CCT CGA GCC ACC ATC ACT GCC 4081
1345 1350 1355

Y R I R H H P E H F S G R P R E
TAC AGG ATC CGC CAT CAT CCC GAG CAC TTC AGT GGG AGA CCT CGA GAA 4129
1360 1365 1370 1375

D R V P H S R N S I T L T N L T
GAT CGG GTG CCC CAC TCT CGG AAT TCC ATC ACC CTC ACC AAC CTC ACT 4177
1380 1385 1390

P G T E Y V V S I V A L N G R E
CCA GGC ACA GAG TAT GTG GTC AGC ATC GTT GCT CTT AAT GGC AGA GAG 4225
1395 1400 1405

FIGURE 1-17

E S P L L I G Q Q S T V S D V P
GAA AGT CCC TTA TTG ATT GGC CAA CAA TCA ACA GTT TCT GAT GTT CCG 4273
1410 1415 1420

R D L E V V A A T P T S L L I S
AGG GAC CTG GAA GTT GTT GCT GCG ACC CCC ACC AGC CTA CTG ATC AGC 4321
1425 1430 1435

W D A P A V T V R Y Y R I T Y G
TGG GAT GCT CCT GCT GTC ACA GTG AGA TAT TAC AGG ATC ACT TAC GGA 4369
1440 1445 1450 1455

E T G G N S P V Q Q E F T V P G S
GAA ACA GGA GGA AAT AGC CCT GTC CAG GAG TTC ACT GTG CCT GGG AGC 4417
1460 1465 1470

K S T A T I S G L K P G V D Y T
AAG TCT ACA GCT ACC ATC AGC GGC CTT AAA CCT GGA GTT GAT TAT ACC 4465
1475 1480 1485

I T V Y A V T G G R G D S P A S S
ATC ACT GTG TAT GCT GTC ACT GGC CGT GGA GAC AGC CCC GCA AGC AGC 4513
1490 1495 1500

FIGURE 1-18

K	P	I	S	I	N	Y	R	T	E	I	D	K	P	S	Q	4561
AAG	CCA	ATT	TCC	ATT	AAT	TAC	CGA	ACA	GAA	ATT	GAC	AAA	CCA	TCC	CAG	
1505						1510					1515					
M	Q	V	T	D	V	Q	D	N	S	I	S	V	K	W	L	4609
ATG	CAA	GTG	ACC	GAT	GTT	CAG	GAC	AAC	AGC	ATT	AGT	GTC	AAG	TGG	CTG	
1520					1525					1530					1535	
P	S	S	S	P	V	T	G	Y	R	V	T	T	T	P	K	4657
CCT	TCA	AGT	TCC	CCT	GTT	ACT	GGT	TAC	AGA	GTA	ACC	ACC	ACT	CCC	AAA	
				1540					1545					1550		
N	G	P	G	P	T	K	T	K	T	A	G	P	D	Q	T	4705
AAT	GGA	CCA	GGA	CCA	ACA	AAA	ACT	AAA	ACT	GCA	GGT	CCA	GAT	CAA	ACA	
				1555				1560					1565			
E	M	T	I	E	G	L	Q	P	T	V	E	Y	V	V	S	4753
GAA	ATG	ACT	ATT	GAA	GGC	TTG	CAG	CCC	ACA	GTG	GAG	TAT	GTG	GTT	AGT	
				1570			1575					1580				

FIGURE 1-19

V Y A Q N P S G E S Q P L V Q T
 GTC TAT GCT CAG AAT CCA AGC GGA GAG AGT CAG CCT CTG GTT CAG ACT 4801
 1585 1590 1595

A V T N I D R P K G L A F T D V
 GCA GTA ACC AAC ATT GAT CGC CCT AAA GGA CTG GCA TTC ACT GAT GTG 4849
 1600 1605 1610 1615

D V D S I K I A W E S P Q G Q V
 GAT GTC GAT TCC ATC AAA ATT GCT TGG GAA AGC CCA CAG GGG CAA GTT 4897
 1620 1625 1630 19/85

S R Y R V T Y S S P E D G I H E
 TCC AGG TAC AGG GTG ACC TAC TCG AGC CCT GAG GAT GGA ATC CAT GAG 4945
 1635 1640 1645

L F P A P D G E E D T A E L Q G
 CTA TTC CCT GCA CCT GAT GGT GAA GAA GAC ACT GCA GAG CTG CAA GGC 4993
 1650 1655 1660

L R P G S E Y T V S V V A L H D
 CTC AGA CCG GGT TCT GAG TAC ACA GTC AGT GTG GTT GCC TTG CAC GAT 5041
 1665 1670 1675

FIGURE 1-20

D	M	E	S	Q	P	L	I	G	T	Q	S	T	A	I	P	
GAT	ATG	GAG	AGC	CAG	CCC	CTG	ATT	GGA	ACC	CAG	TCC	ACA	GCT	ATT	CCT	5089
1680				1685				1690						1695		

A	P	T	D	L	K	F	T	Q	V	T	P	T	S	L	S	
GCA	CCA	ACT	GAC	CTG	AAG	TTC	ACT	CAG	GTC	ACA	CCC	ACA	AGC	CTG	AGC	5137
				1700				1705						1710		

A	Q	W	T	P	P	N	V	Q	L	T	G	Y	R	V	R	
GCC	CAG	TGG	ACA	CCA	CCC	AAT	GTT	CAG	CTC	ACT	GGA	TAT	CGA	GTG	CGG	5185
			1715					1720					1725			

V	T	P	K	E	K	T	G	P	M	K	E	I	N	L	A	
GTG	ACC	CCC	AAG	GAG	AAG	ACC	GGA	CCA	ATG	AAA	GAA	ATC	AAC	CTT	GCT	5233
		1730					1735					1740				

P	D	S	S	S	V	V	V	S	G	L	M	V	A	T	K	
CCT	GAC	AGC	TCA	TCC	GTG	GTT	GTA	TCA	GGA	CTT	ATG	GTG	GCC	ACC	AAA	5281
	1745					1750				1755						

FIGURE 1-21

Y E V S V Y A L K D T L T S R P
TAT GAA GTG AGT GTC TAT GCT CTT AAG GAC ACT TTG ACA AGC AGA CCA
1760 1765 1770 1775 5329

A Q G V V T T L E N V S P P R R
GCT CAG GGT GTT GTC ACC ACT CTG GAG AAT GTC AGC CCA CCA AGA AGG
1780 1785 1790 5377

A R V T D A T E T T I T I S W R
GCT CGT GTG ACA GAT GCT ACT GAG ACC ACC ATC ACC ATT AGC TGG AGA
1795 1800 1805 5425

T K T E T I T G F Q V D A V P A
ACC AAG ACT GAG ACG ATC ACT GGC TTC CAA GTT GAT GCC GTT CCA GCC
1810 1815 1820 5473

N G Q T P I Q R T I K P D V R S
AAT GGC CAG ACT CCA ATC CAG AGA ACC ATC AAG CCA GAT GTC AGA AGC
1825 1830 1835 5521

Y T I T G L Q P G T D Y K I Y L
TAC ACC ATC ACA GGT TTA CAA CCA GGC ACT GAC TAC AAG ATC TAC CTG
1840 1845 1850 1855 5569

FIGURE 1-22

Y T L N D N A R S S P V V I D A
TAC ACC TTG AAT GAC AAT GCT CGG AGC TCC CCT GTG GTC ATC GAC GCC 5617
1860 1865 1870

S T A I D A P S N L R F L A T T
TCC ACT GCC ATT GAT GCA CCA TCC AAC CTG CGT TTC CTG GCC ACC ACA 5665
1875 1880 1885

P N S L L V S W Q P P R A R I T
CCC AAT TCC TTG CTG GTA TCA TGG CAG CCG CCA CGT GCC AGG ATT ACC 5713
1890 1895 1900 22/86

G Y I I K Y E K P G S P P R E V
GGC TAC ATC ATC AAG TAT GAG AAG CCT GGG TCT CCT CCC AGA GAA GTG 5761
1905 1910 1915

V P R P R P G V T E A T I T G L
GTC CCT CGG CCC CGC CCT GGT GTC ACA GAG GCT ACT ATT ACT GGC CTG 5809
1920 1925 1930 1935

FIGURE 1-23

E P G T E Y T I Y V I A L K N N
GAA CCG GGA ACC GAA TAT ACA ATT TAT GTC ATT GCC CTG AAG AAT AAT
1940 1945 1950 5857

Q K S E P L I G R K K T D E L P
CAG AAG AGC GAG CCC CTG ATT GGA AGG AAA AAG ACA GAC GAG CTT CCC
1955 1960 1965 5905

Q L V T L P H P N L H G P E I L
CAA CTG GTA ACC CTT CCA CAC CCC AAT CTT CAT GGA CCA GAG ATC TTG
1970 1975 1980 5953
23/85

D V P S T V Q K T P F V T H P G
GAT GTT CCT TCC ACA GTT CAA AAG ACC CCT TTC GTC ACC CAC CCT GGG
1985 1990 1995 6001

Y D T G N G I Q L P G T S G Q Q
TAT GAC ACT GGA AAT GGT ATT CAG CTT CCT GGC ACT TCT GGT CAG CAA
2000 2005 2010 2015 6049

P S V G Q Q M I F E E H G F R R
CCC AGT GTT GGG CAA CAA ATG ATC TTT GAG GAA CAT GGT TTT AGG CGG
2020 2025 2030 6097

FIGURE 1-24

T T P P T T T A T P I R H R P P
ACC ACA CCG CCC ACA ACG GCC ACC CCC ATA AGG CAT AGG CCA AGA CCA 6145
2035 2040 2045

Y P P N V G Q E A L S Q T T I S
TAC CCG CCG AAT GTA GGA CAA GAA GCT CTC TCT CAG ACA ACC ATC TCA 6193
2050 2055 2060

W A P F Q D T S E Y I I S C H P
TGG GCC CCA TTC CAG GAC ACT TCT GAG TAC ATC ATT TCA TGT CAT CCT 6241
2065 2070 2075

V G T D E E P L Q F R V P G T S
GTT GGC ACT GAT GAA GAA CCC TTA CAG TTC AGG GTT CCT GGA ACT TCT 6289
2080 2085 2090 2095

T S A T L T G L T R G A T Y N I
ACC AGT GCC ACT CTG ACA GGC CTC ACC AGA GGT GCC ACC TAC AAC ATC 6337
2100 2105 2110

FIGURE 1-25

I V E A L K D Q Q R H K V R E E
ATA GTG GAG GCA CTG AAA GAC CAG CAG AGG CAT AAG GTT CGG GAA GAG 6385
2115 2120 2125

V V T V G N S V N E G L N Q P T
GTT GTT ACC GTG GGC AAC TCT GTC AAC GAA GGC TTG AAC CAA CCT ACG 6433
2130 2135 2140

D D S C F D P Y T V S H Y A V G
GAT GAC TCG TGC TTT GAC CCC TAC ACA GTT TCC CAT TAT GCC GTT GGA 6481
2145 2150 2155 25/89

D E W E R M S E S G F K L L C Q
GAT GAG TGG GAA CGA ATG TCT GAA TCA GGC TTT AAA CTG TTG TGC CAG 6529
2160 2165 2170 2175

C L G F G S G H F R C D S S R W
TGC TTA GGC TTT GGA AGT GGT CAT TTC AGA TGT GAT TCA TCT AGA TGG 6577
2180 2185 2190

C H D N G V N Y K I G E K W D R
TGC CAT GAC AAT GGT GTG AAC TAC AAG ATT GGA GAG AAG TGG GAC CGT 6625
2195 2200 2205

FIGURE 1-26

Q G E N G Q M M S C T C L G N G
CAG GGA GAA AAT GGC CAG ATG ATG AGC TGC ACA TGT CTT GGG AAC GGA 6673
2210 2215 2220

K G E F K C D P H E A T C Y D D
AAA GGA GAA TTC AAG TGT GAC CCT CAT GAG GCA ACG TGT TAC GAT GAT 6721
2225 2230 2235 20/86

G K T Y H V G E Q W Q K E Y L G
GGG AAG ACA TAC CAC GTA GGA GAA CAG TGG CAG AAG GAA TAT CTC GGT 6769
2240 2245 2250 2255

A I C S C T C F G G Q R G W R C
GCC ATT TGC TCC TGC ACA TGC TTT GGA GGC CAG CGG GGC TGG CGC TGT 6817
2260 2265 2270

D N C R R P G G E P S P E G T T
GAC AAC TGC CGC AGA CCT GGG GGT GAA CCC AGT CCC GAA GGC ACT ACT 6865
2275 2280 2285

FIGURE 1-27

G	Q	S	Y	N	Q	Y	S	Q	R	Y	H	Q	R	T	N	6913
GGC	CAG	TCC	TAC	AAC	CAG	TAT	TCT	CAG	AGA	TAC	CAT	CAG	AGA	ACA	AAC	
2290							2295					2300				
T	N	V	N	C	P	I	E	C	F	M	P	L	D	V	Q	6961
ACT	AAT	GTT	AAT	TGC	CCA	ATT	GAG	TGC	TTC	ATG	CCT	TTA	GAT	GTA	CAG	
2305						2310					2315					
A	D	R	E	D	S	R	E									7009
GCT	GAC	AGA	GAA	GAT	TCC	CGA	GAG	TAA	ATC	ATC	TTT	CCA	ATC	CAG	AGG	
2320					2325					2330					2335	
AAC	AAG	CAT	GTC	TCT	CTG	CCA	AGA	TCC	ATC	TAA	ACT	GGA	GTG	ATG	TTA	7057
				2340				2345								
GCA	GAC	CCA	GCT	TAG	AGT	TCT	TCT	TTC	TTT	CTT	AAG	CCC	TTT	GCT	CTG	7105
			2355					2360					2365			
GAG	GAA	GTT	CTC	CAG	CTT	CAG	CTC	AAC	TCA	CAG	CTT	CTC	CAA	GCA	TCA	7153
		2370					2375					2380				

FIGURE 1-28

CCC TGG GAG TTT CCT GAG GGT TTT CTC ATA AAT GAG GGC TGC ACA TTG	7201
2385	2390
2395	
CCT GTP CTG CTT CGA AGT ATT CAA TAC CGC TCA GTA TTT TAA ATG AAG	7249
2400	2405
2410	2415
TGA TTC TAA GAT TTG GTT TGG GAT CAA TAG GAA AGC ATA TGC AGC CAA	7297
2420	2425
2430	
CCA AGA TGC AAA TGT TTT GAA ATG ATA TGA CCA AAA TTT TAA GTA GGA	7345
2435	2440
2445	
AAG TCA CCC AAA CAC TTC TGC TTT CAC TTA AGT GTC TGG CCC GCA ATA	7393
2450	2455
2460	
CTG TAG GAA CAA GCA TGA TCT TGT TAC TGT GAT ATT TTA AAT ATC CAC	7441
2465	2470
2475	
AGT ACT CAC TTT TTC CAA ATG ATC CTA GTA ATT GCC TAG AAA TAT CTT	7489
2480	2485
2490	2495

FIGURE 1-29

TCT CTT ACC TGT TAT TTA TCA ATT TTT CCC AGT ATT TTT ATA CGG AAA	7537
2500	2505
2510	
AAA TTG TAT TGA AAA CAC TTA GTA TGC AGT TGA TAA GAG GAA TTT GGT	7585
2515	2520
2525	
ATA ATT ATG GTG GGT GAT TAT TTT TTA TAC TGT ATG TGC CAA AGC TTT	7633
2530	2535
2540	
ACT ACT GTG GAA AGA CAA CTG TTT TAA TAA AAG ATT TAC ATT CCA CAA	7681
2545	2550
2555	
AAAAAAAAAAAA AAAAAAAAAAAAA AAAA	7705

FIGURE 2A

PAIR 1

#1 5'- AATTCAATGCGAGCAGCAAGCAATGTTACGCCCCAGTCCCGGTGGCTGTCAGTCAAGCAAGCCCGTT -3'
#2 3'- GTATACGTCCTCGTCTTACCAAGTCGGGTCAGGGGCCACCGACAGTCACTTCTCGGGCCAACAATA -5'

PAIR 2

#3 5'- GTTATGACATGGAACAACATATCAGATAAATCAACAGTGGAGCGGACCTACCTAGGTAATGTGTTG -3'
#4 3'- CTGTTACCTTTTGTGATAGTCTATTTAGTTGTCACCCCTCGCCTGGATGATCCATT -5'

PAIR 3

#5 5'- GTTGTACTTGTATGAGGAAGCCGAGGTTTAACTGCGAAAGTAAACCTGAAGCT -3'
#6 3'- ACACACCAACATGAACAATACCTCCTCGGCTCCAAATTTGACGCTTTCATTTGGACTTCGACTTCTCT -5'

PAIR 4

#7 5'- GAAGAGACTTGTGACAGTACACTGGGAACACTTACCGAGTGGGTGACACTTATGAGCGTCCCTAA -3'
#8 3'- GAACGAACCTGTTCATGTGACCCCTTGTGAATGGCTCACCCACTGTGAATACTCGCAG -5'

FIGURE 2B

PAIR 5

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#9 5'- GACTCCATGATCTGGGACTGTACCTGCATCGGGGCTGGGCGAGGAGATAAGCTGTACC -3'
 #10 GATTTCGAGGTACTAGACCCCTGACATGGACGTAGCCCCGACCCGCTCCCTTATTTC -5'

PAIR 6

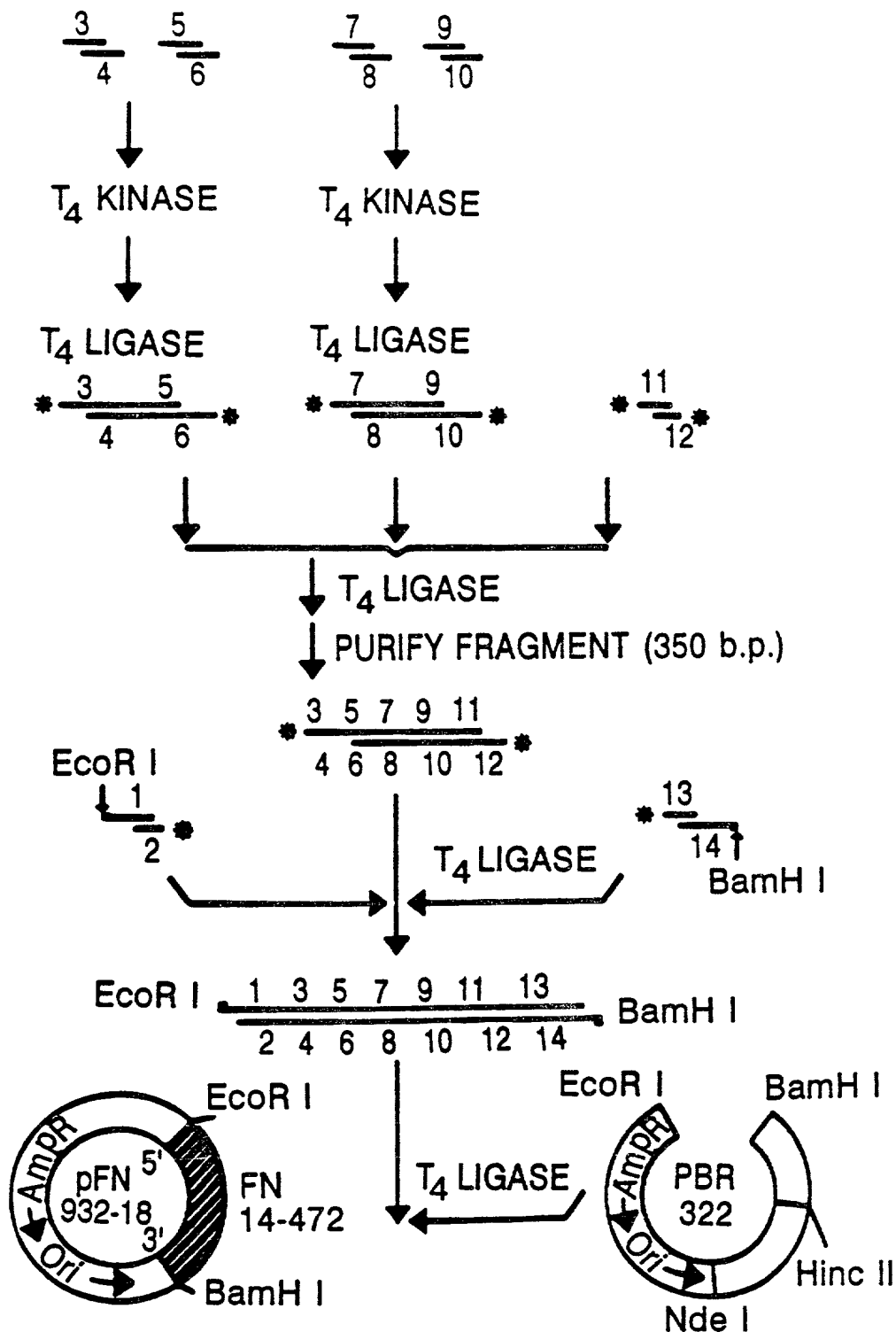
#11 5'- ATGCCCAACGCTGCCCATGAAGGGGTCAGTCCTACCAGATTGGTGACACCTGGAGGAGACCATGAGACT -3'
 #12 3'- GACATGGTAGCGTTTGCGACGGTACTTCCCCCAGTCAGGATGGTCTAACCACTGTGGACCTCCTGTGTA CTGTGACCAACCAA-5'

PAIR 7

#13 5'- GGTGGTTACATGTTAGAGTGTGTGTCCTTGGTAATGGAAGAAGAGATGACCTGCAAGCCCATAGCTGAG -3'
 #14 3'- TGTACAATCTCACACACAGAAACCATTAACCTTTTCCCTTAACCTGGACGTTGGGGTATCGACTCCCTAG -5'

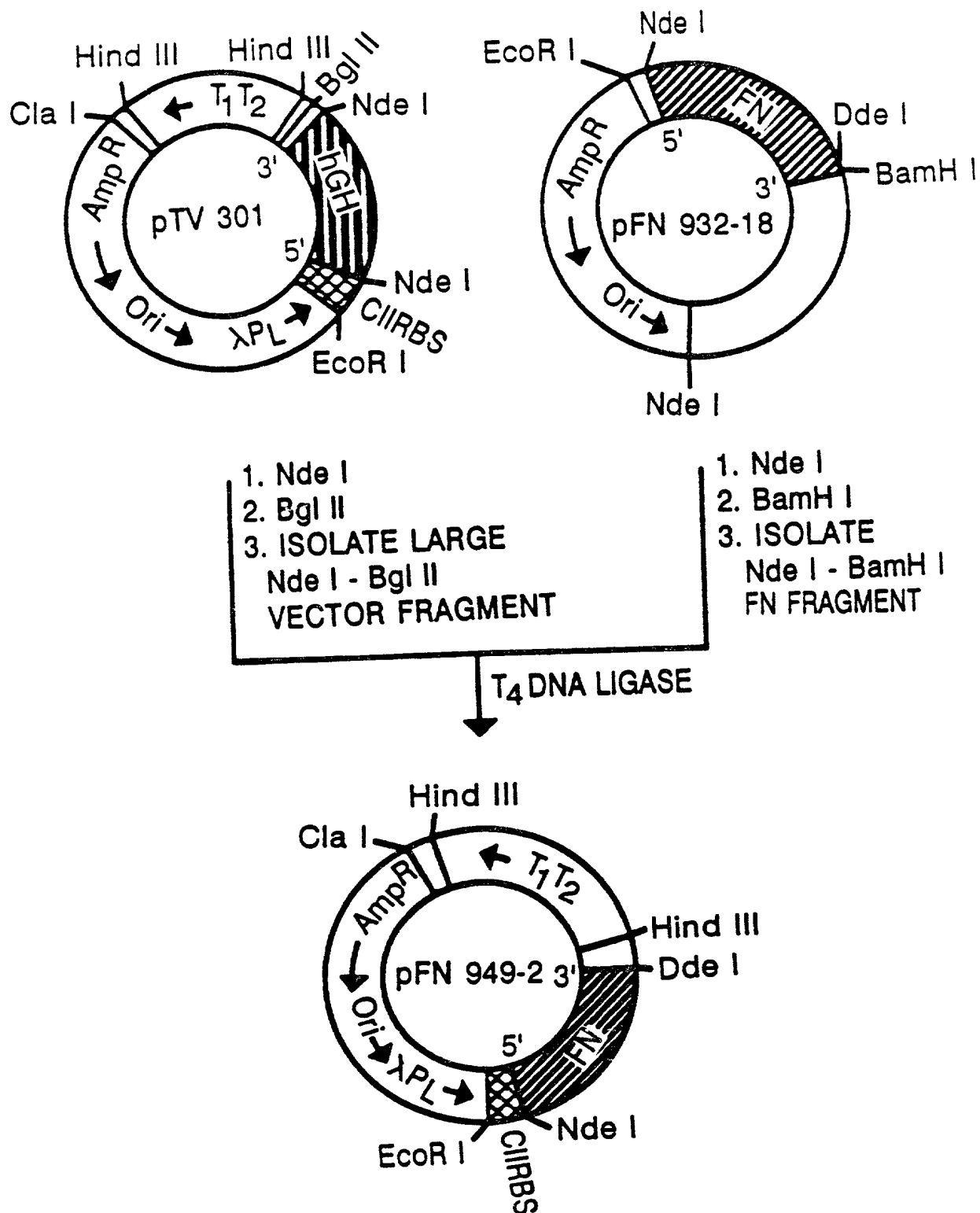
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FIGURE 3

SYNTHESIS OF F.N. GENE (14-472 b.p.)



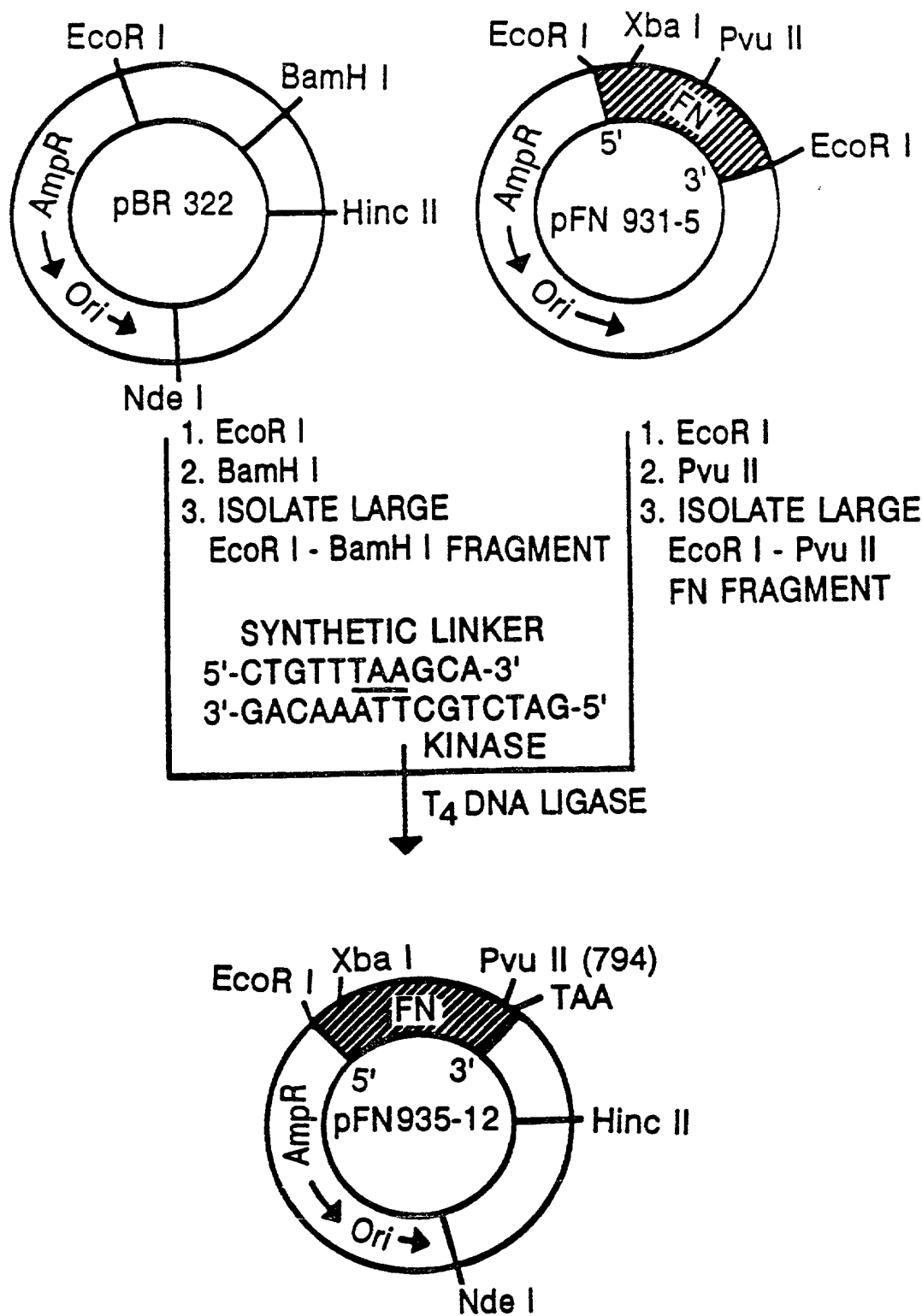
002270-12626460

33/86
FIGURE 4



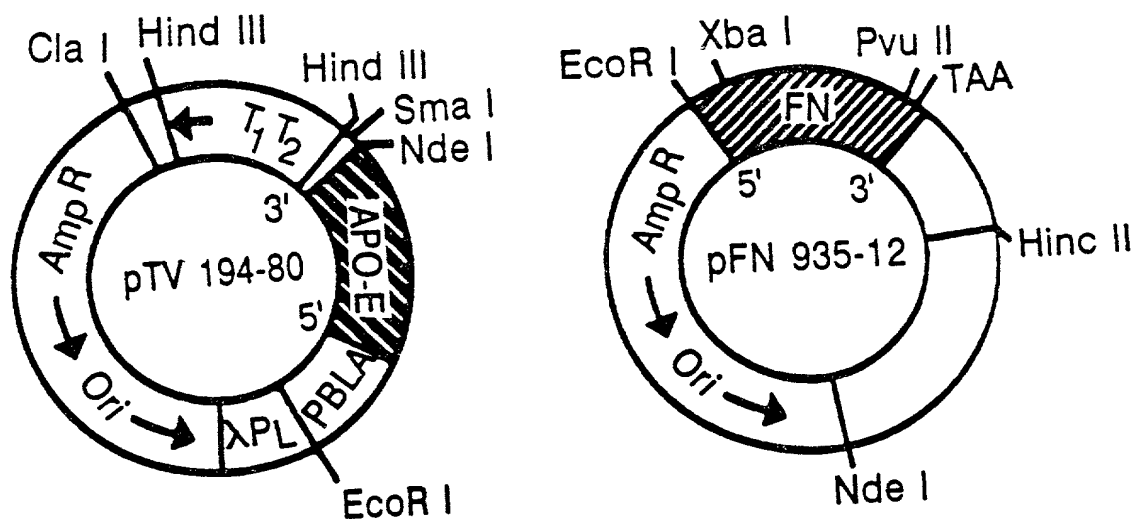
00220 T 2626460

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FIGURE 5



002210-1655460

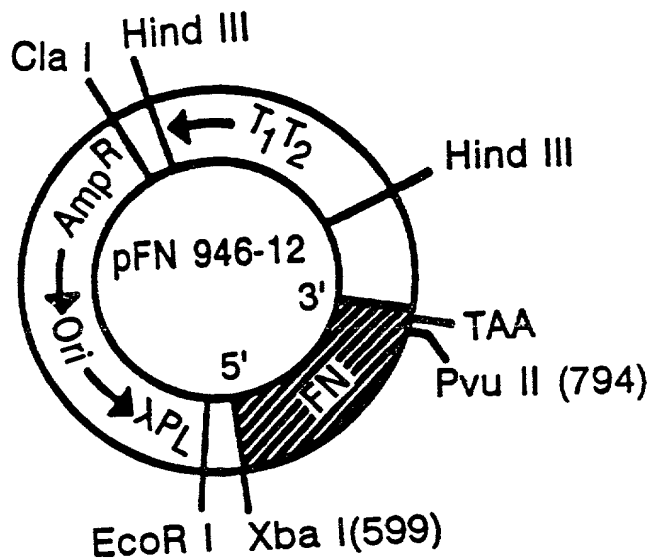
35/86
FIGURE 6



1. EcoR I
2. Sma I
3. ISOLATE LARGE
EcoR I - Sma I FRAGMENT

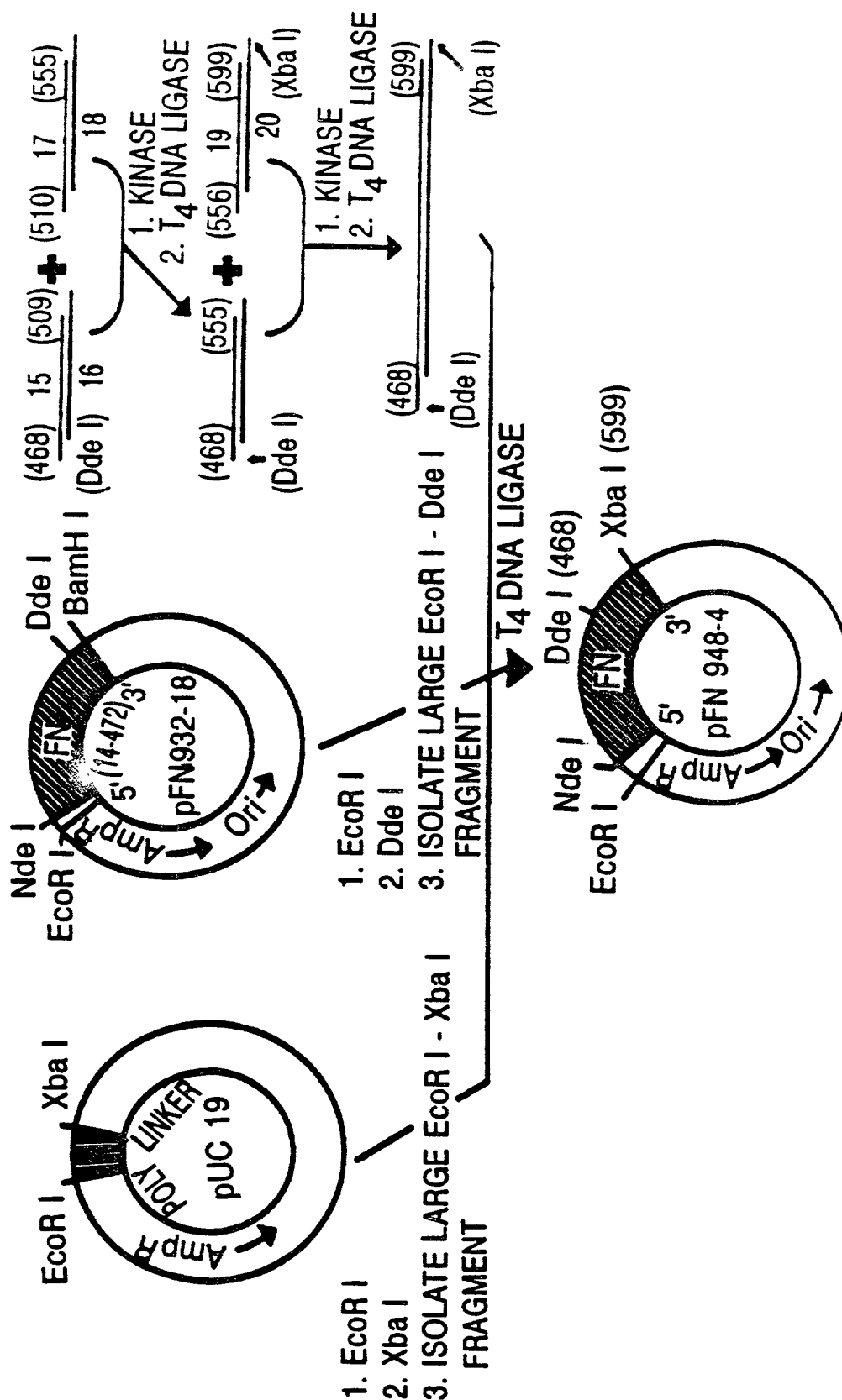
1. EcoR I
2. Hinc II
3. ISOLATE
EcoR I - Hinc II
FN FRAGMENT

T₄ DNA LIGASE

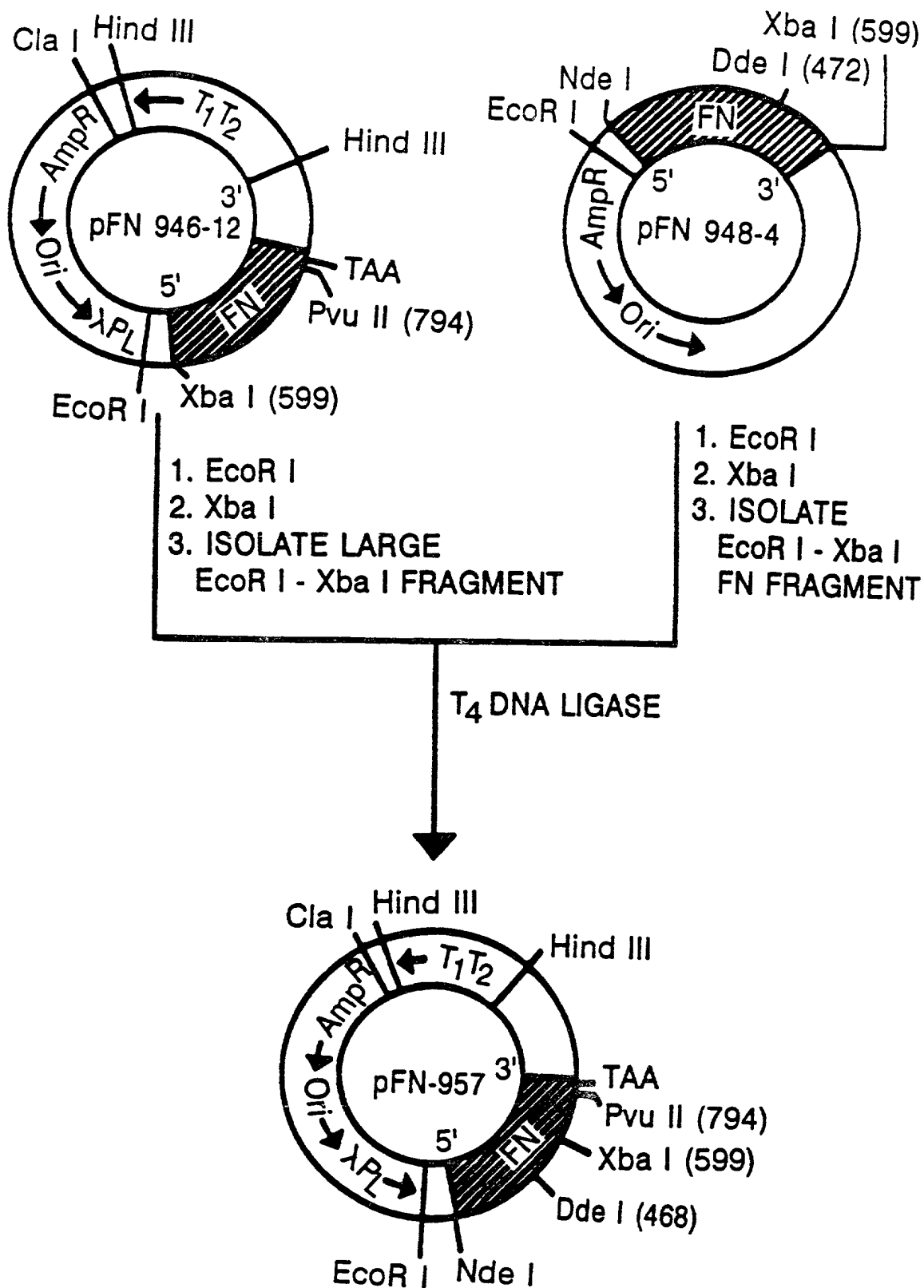


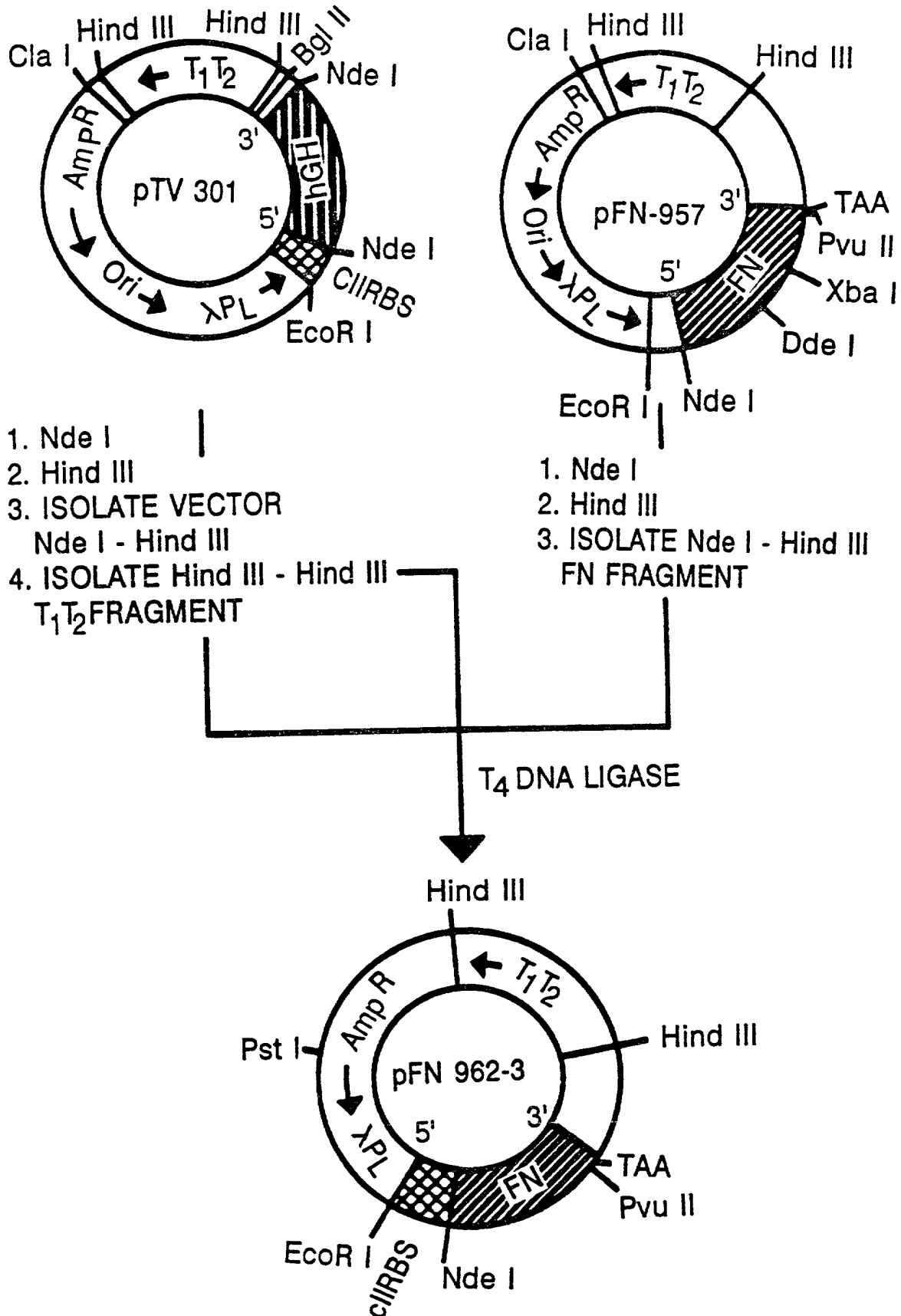
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FIGURE 7

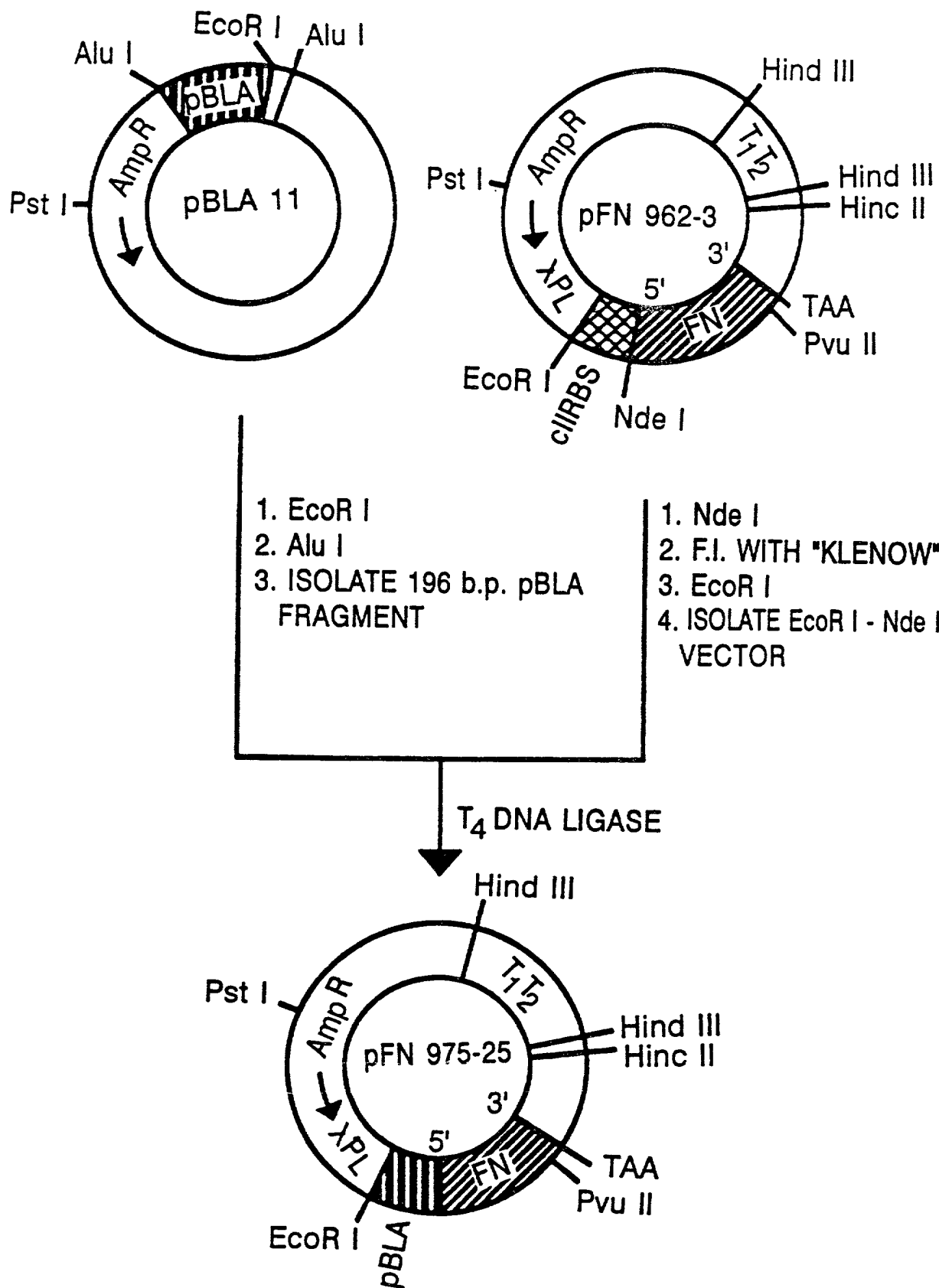


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FIGURE 8





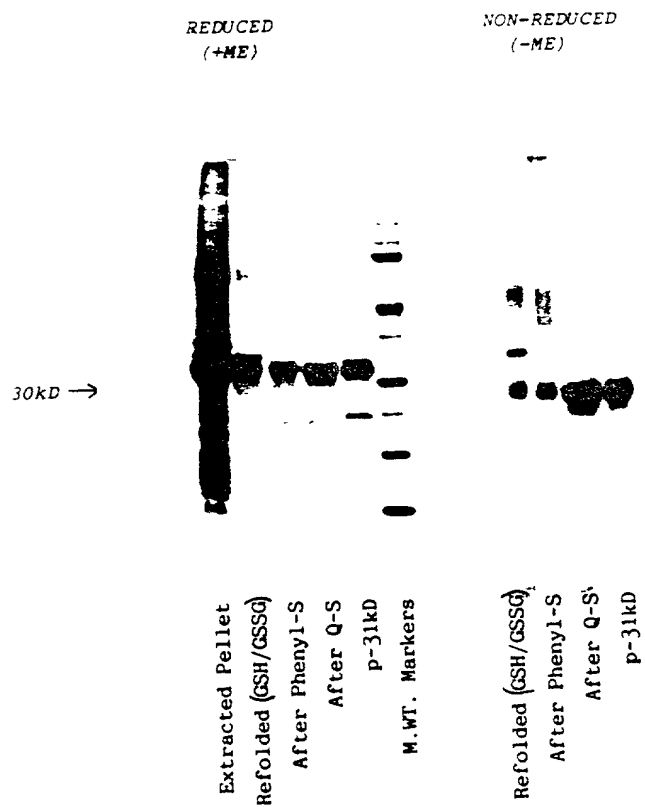
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FIGURE 10



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FIGURE 11



ODx10²

FIGURE 12

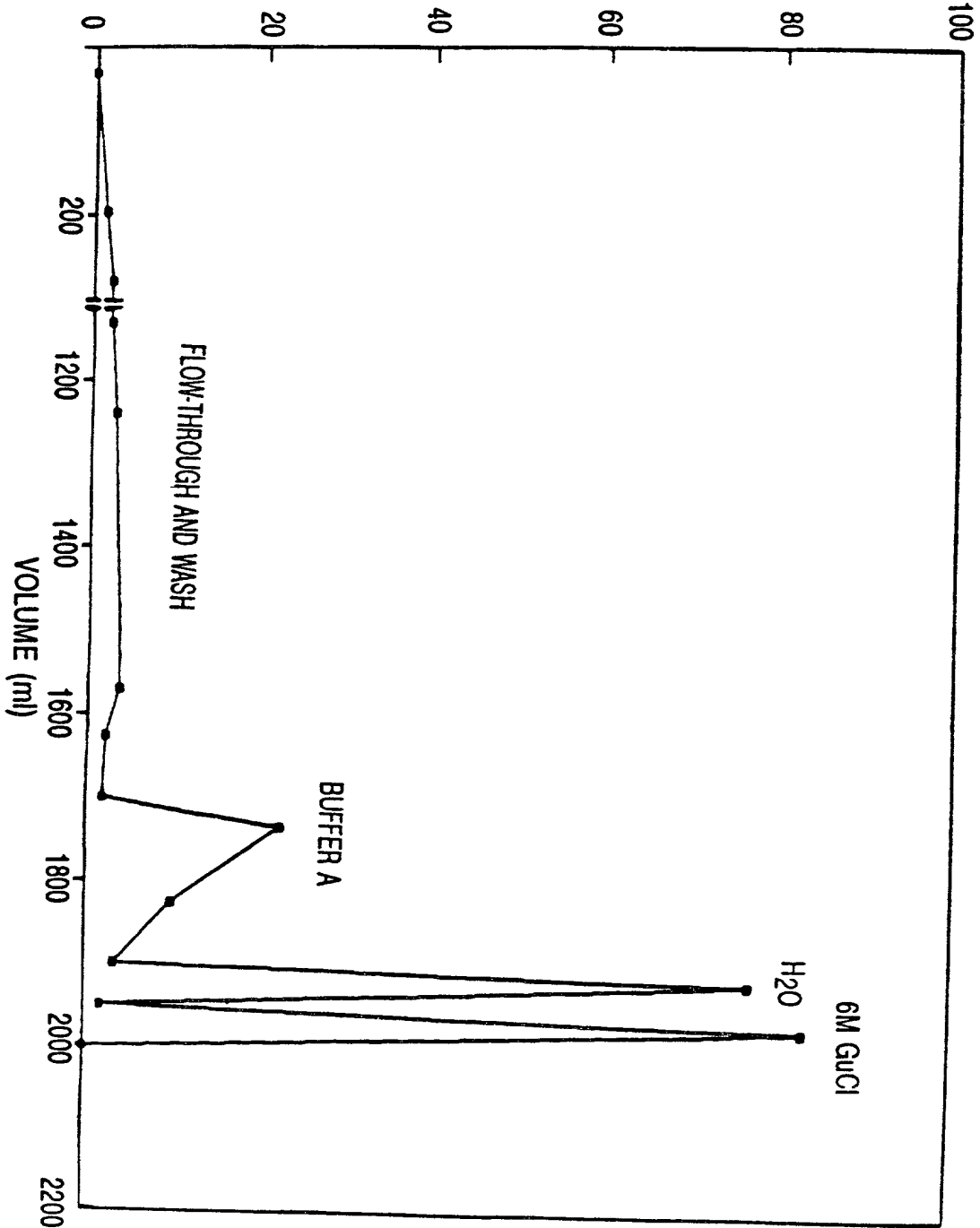
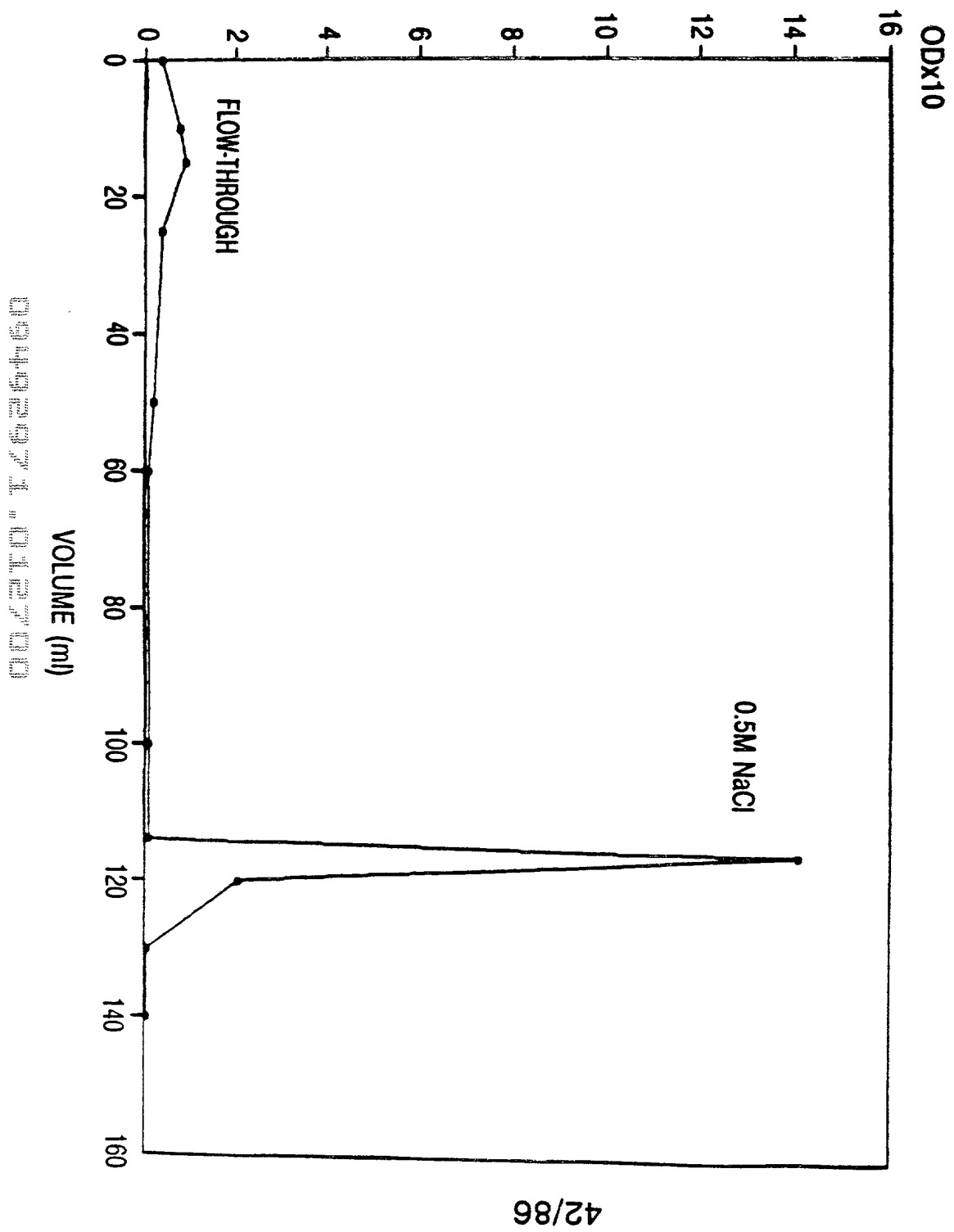
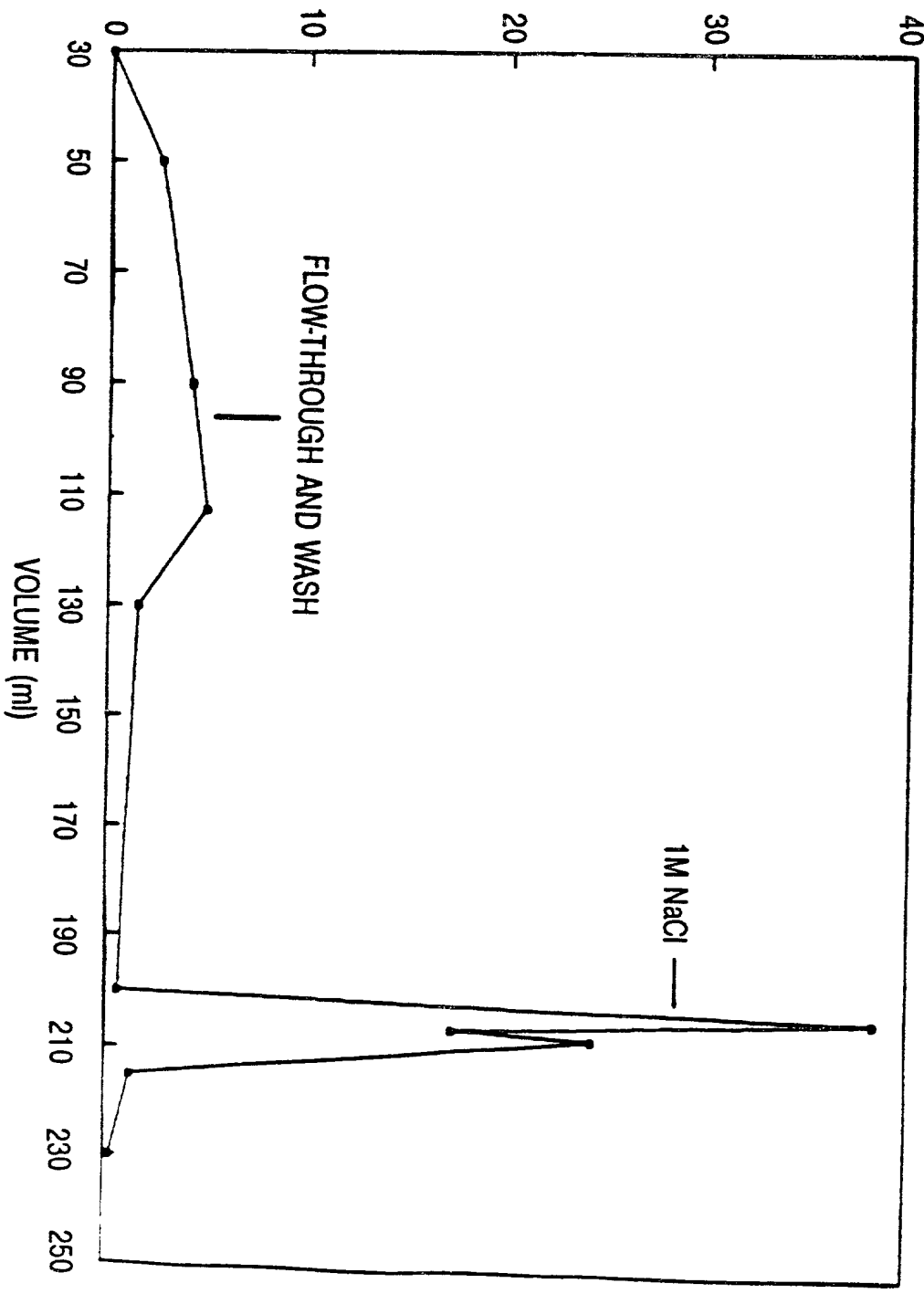


FIGURE 13



ODx10²

FIGURE 14

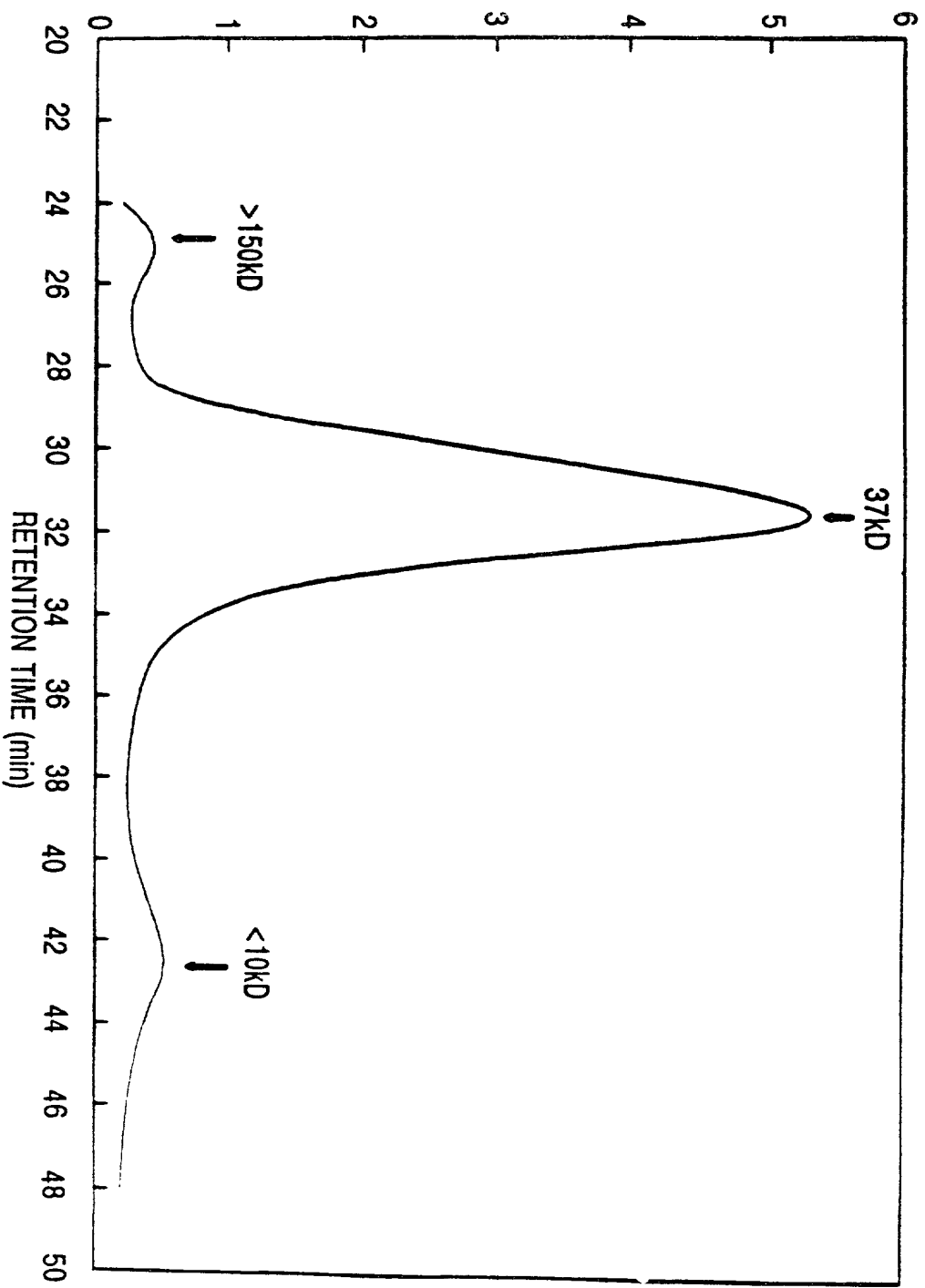


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ODx10²

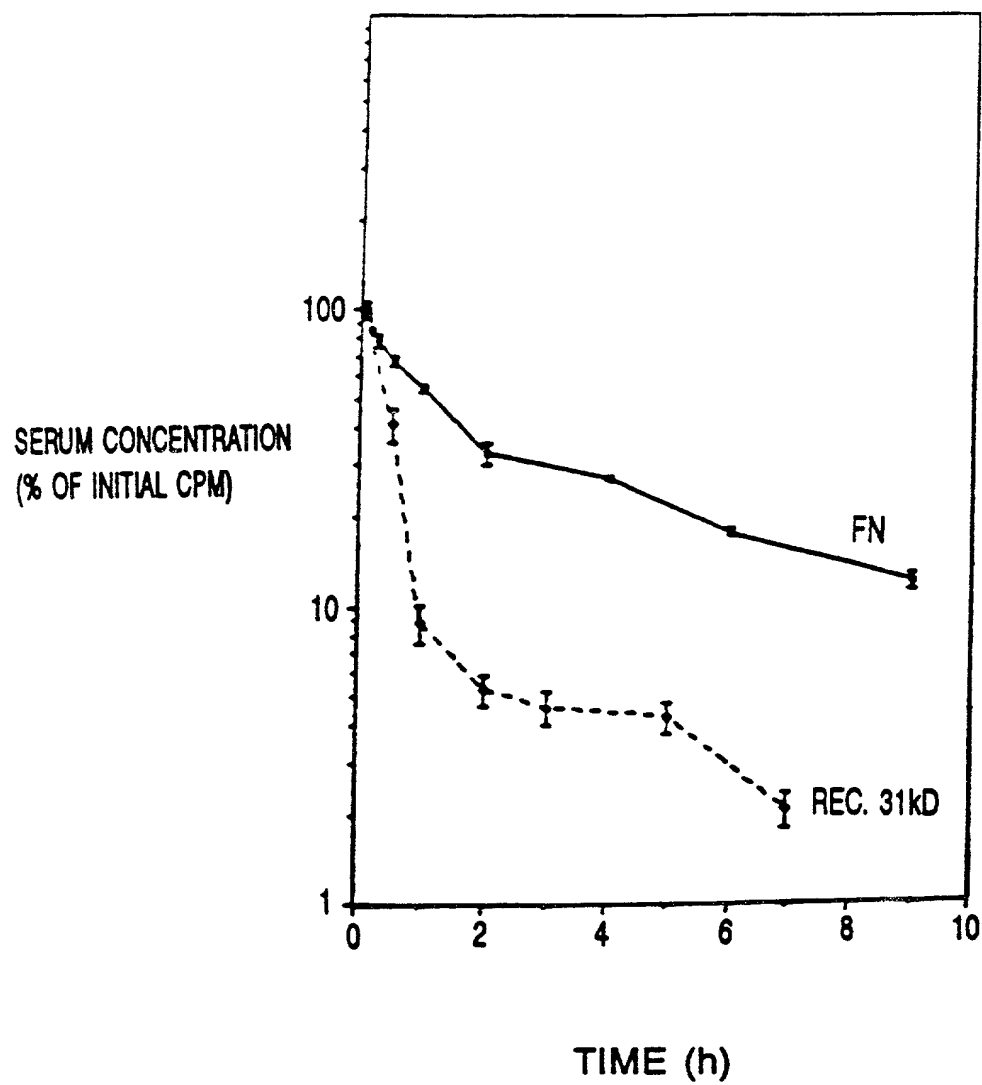
FIGURE 15



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FIGURE 16



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FIGURE 17

I = Binding while clot formation
II = Binding to preformed Fibrin clot

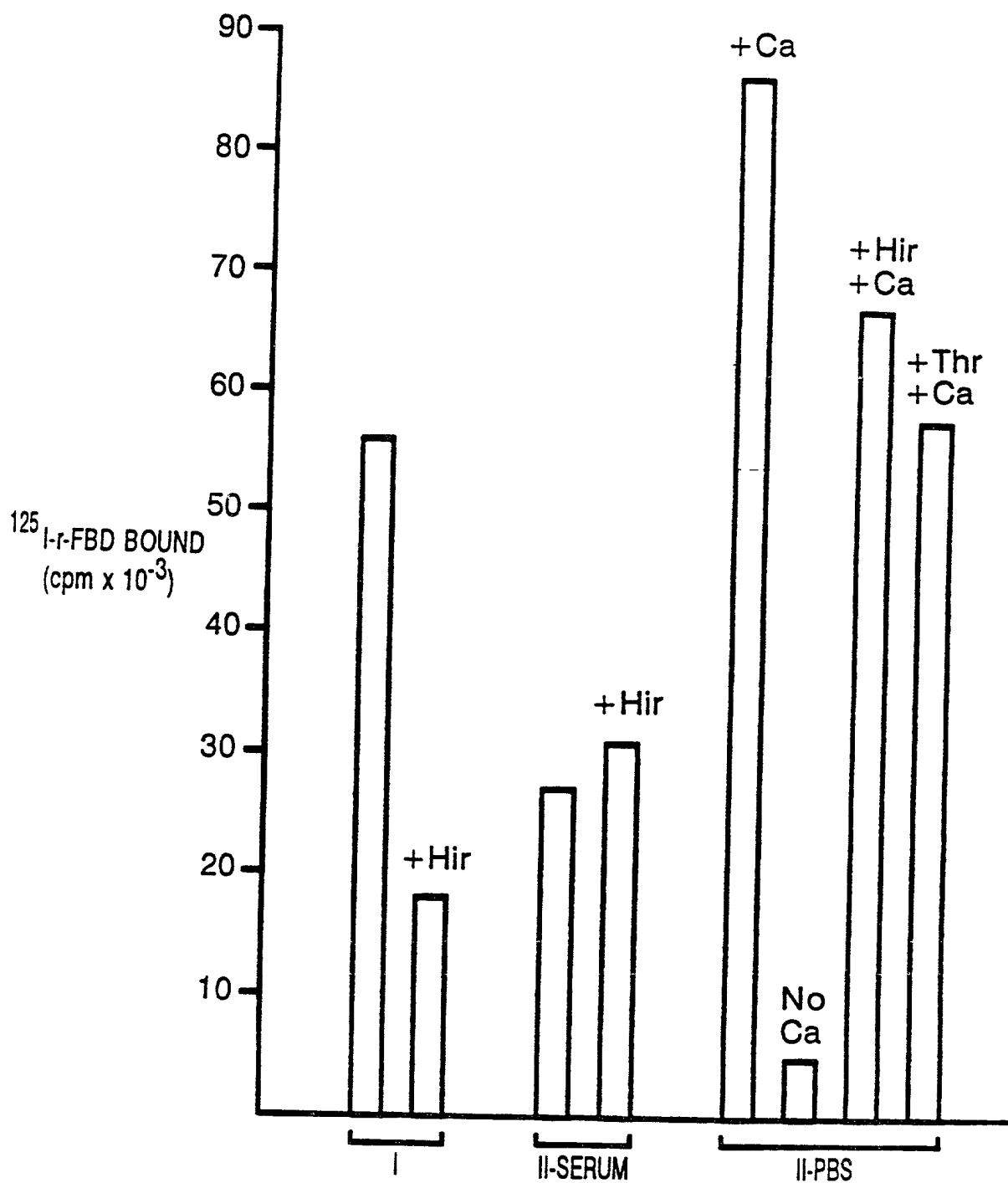
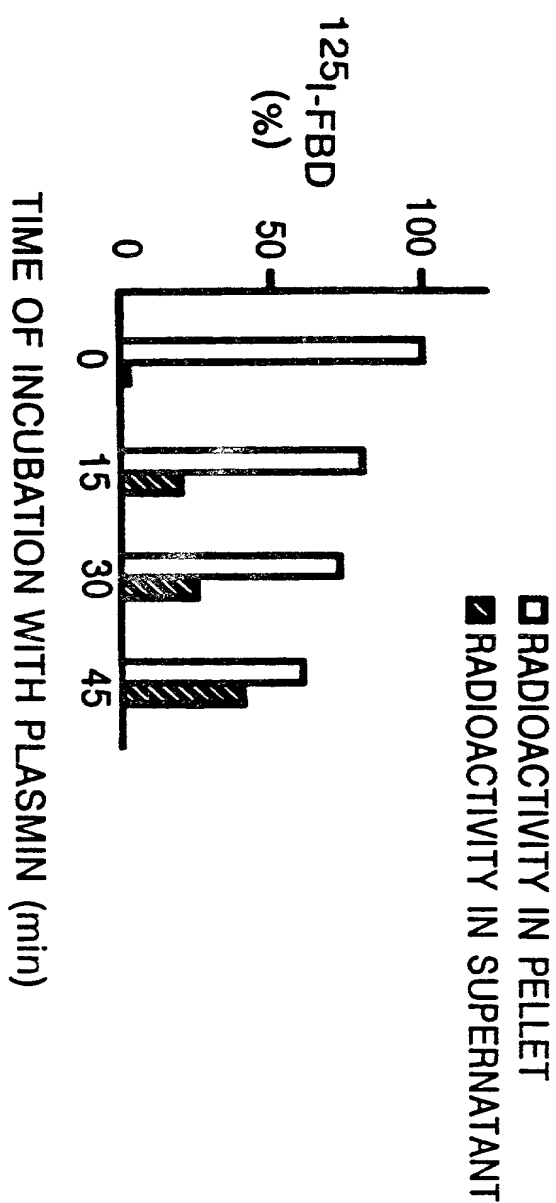
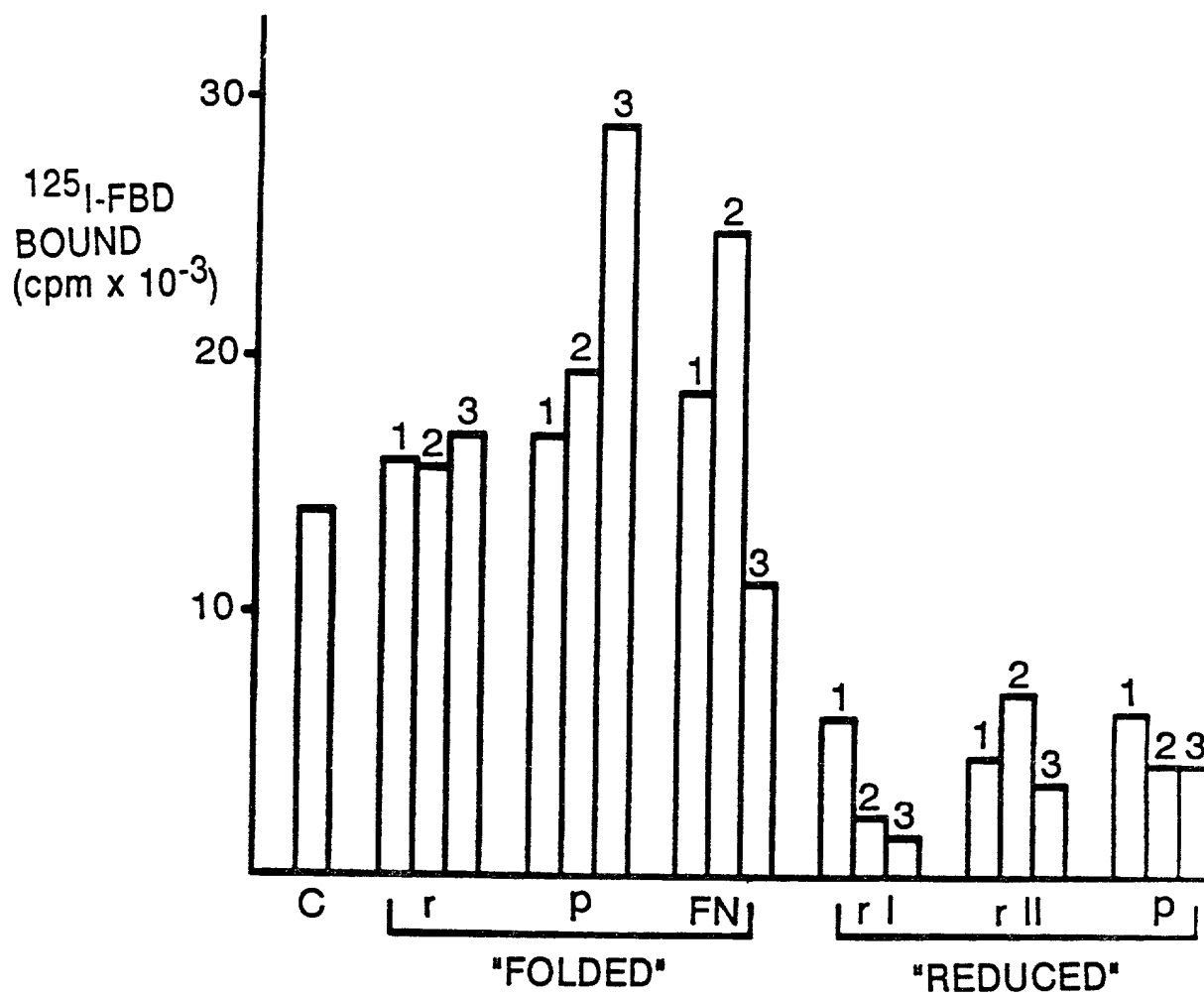


FIGURE 18



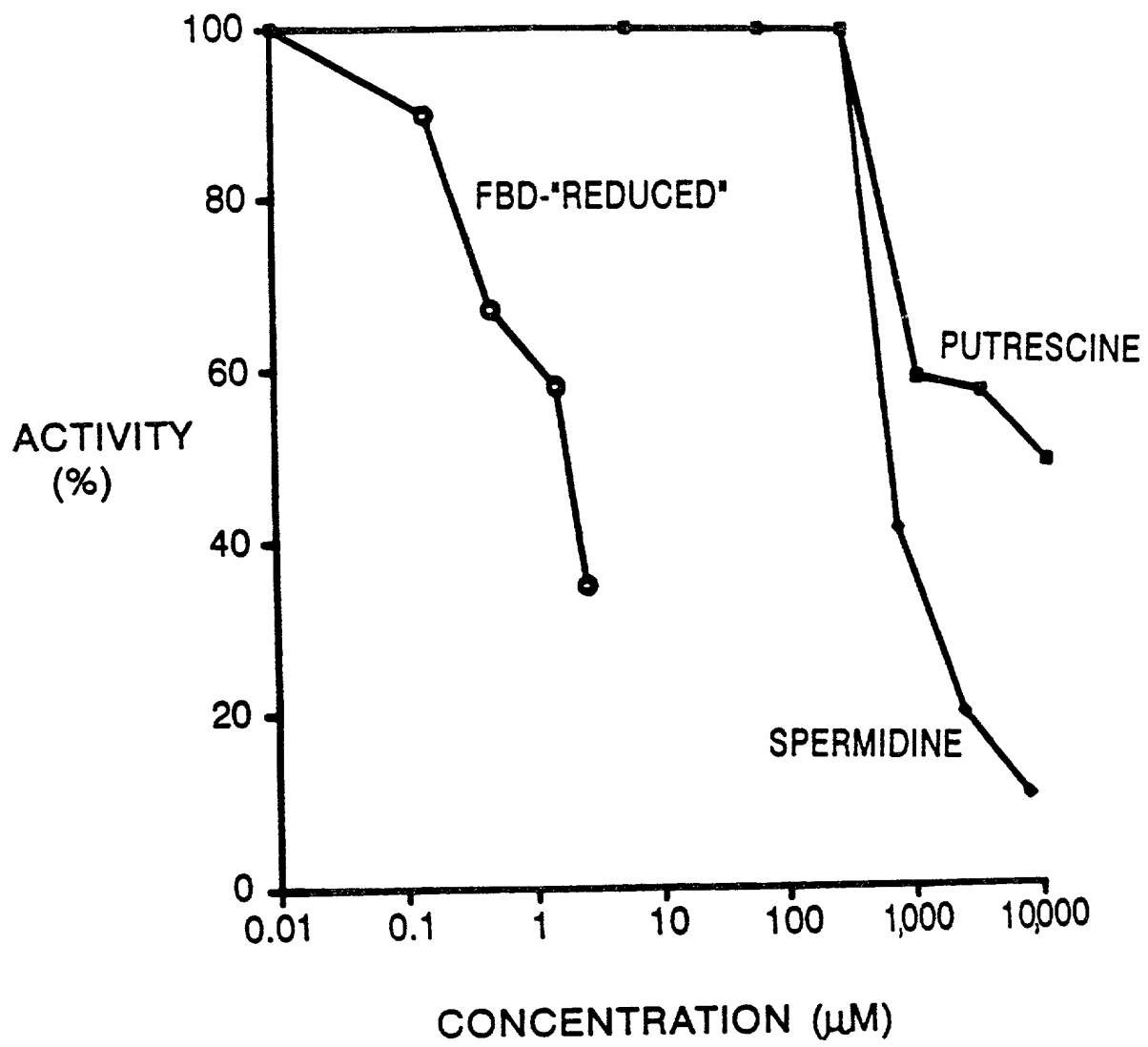
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FIGURE 19

1 - 0.3 μ M Unlabelled competitors
2 - 1.0 μ M " "
3 - 3.0 μ M " "

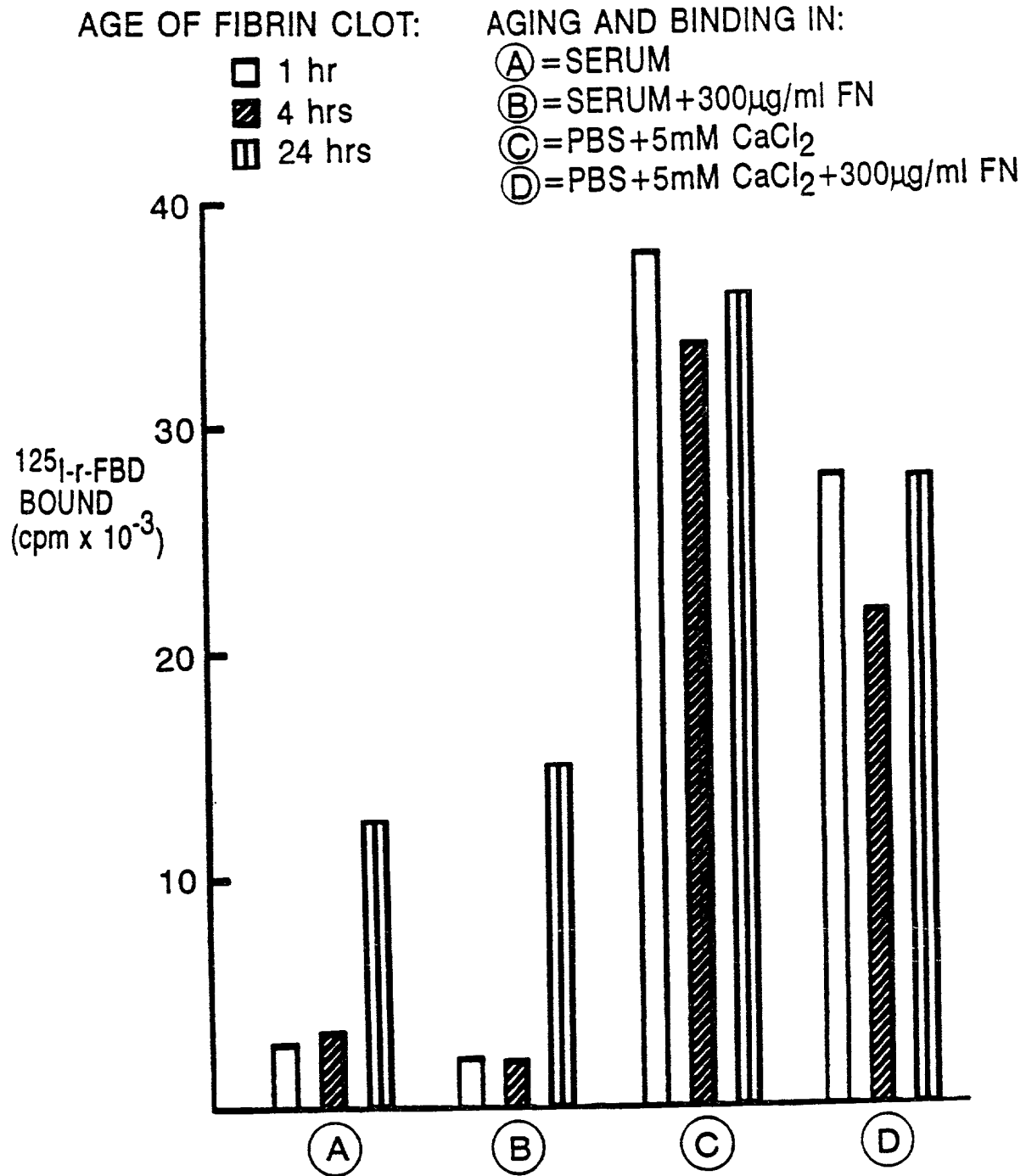


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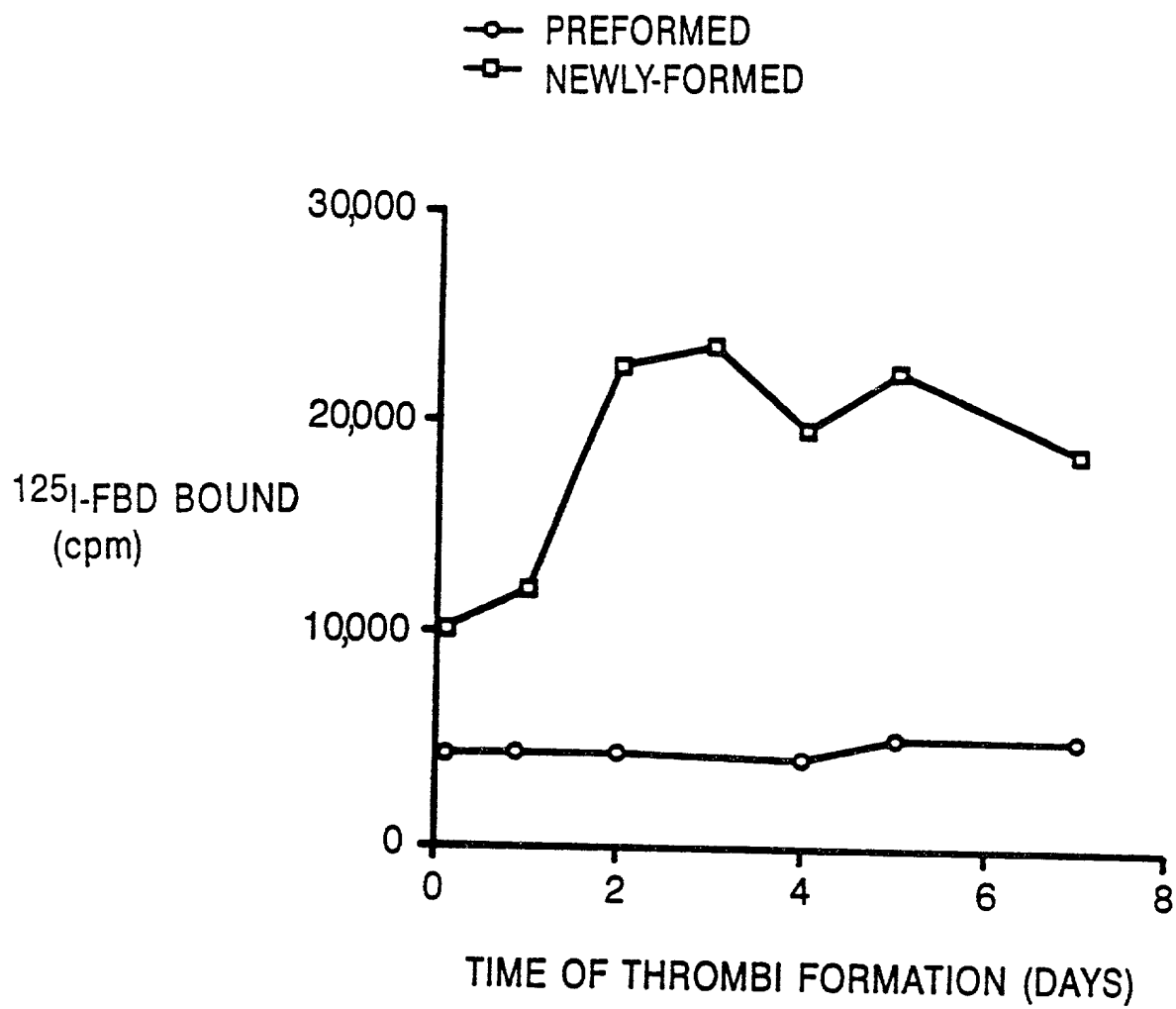
FIGURE 20

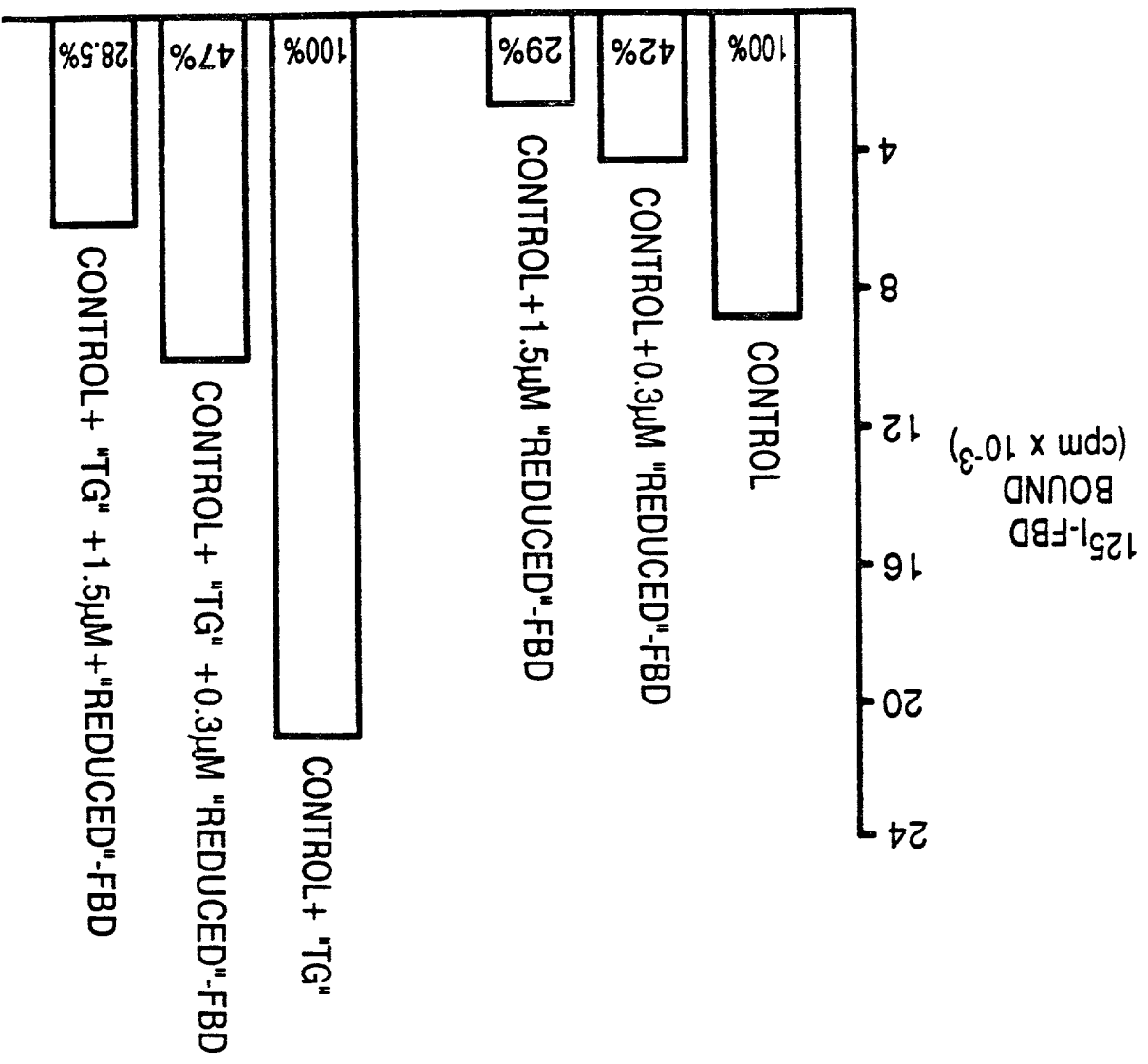


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FIGURE 21



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FIGURE 22

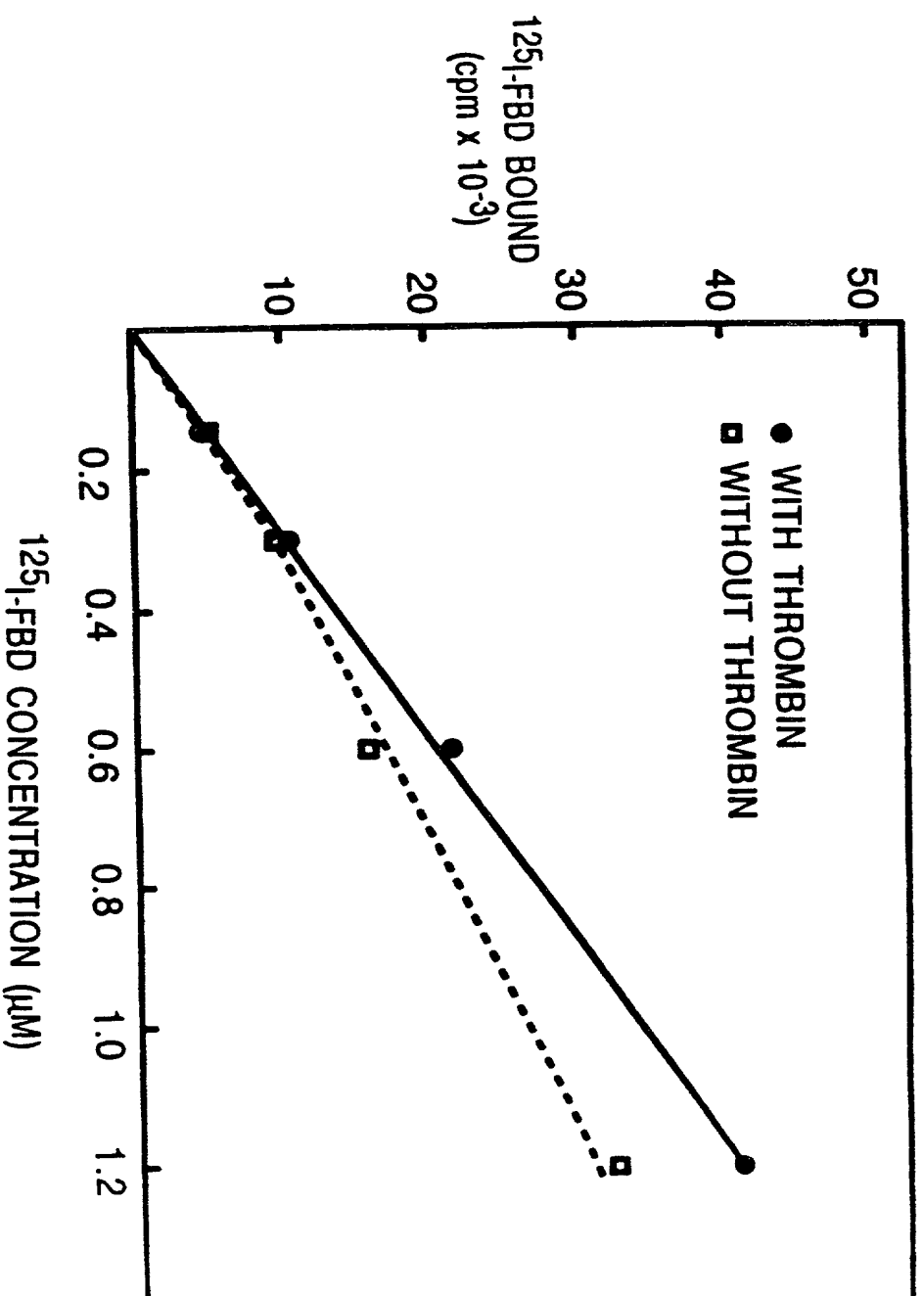




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FIGURE 23

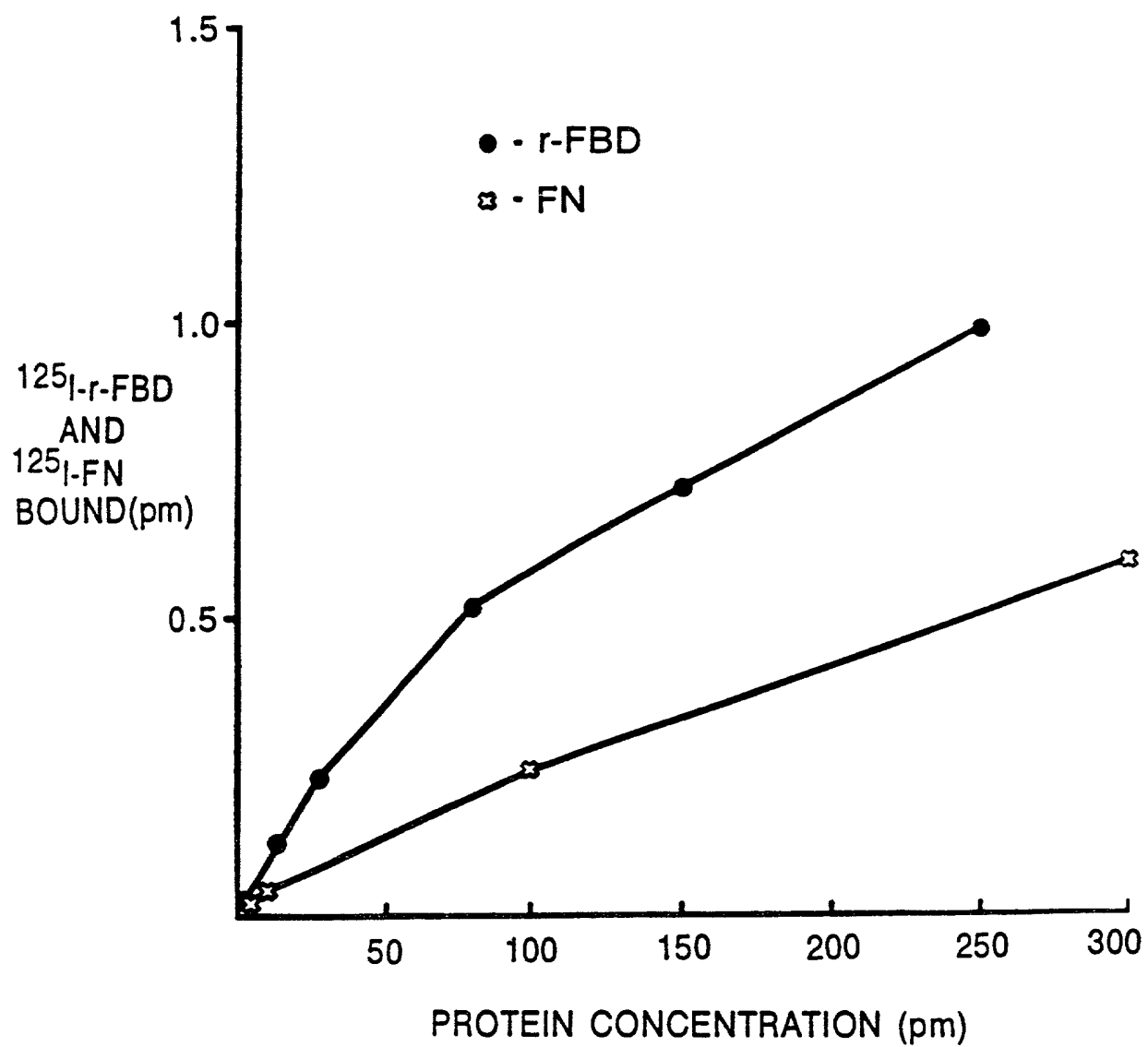
09492971.012700

FIGURE 24



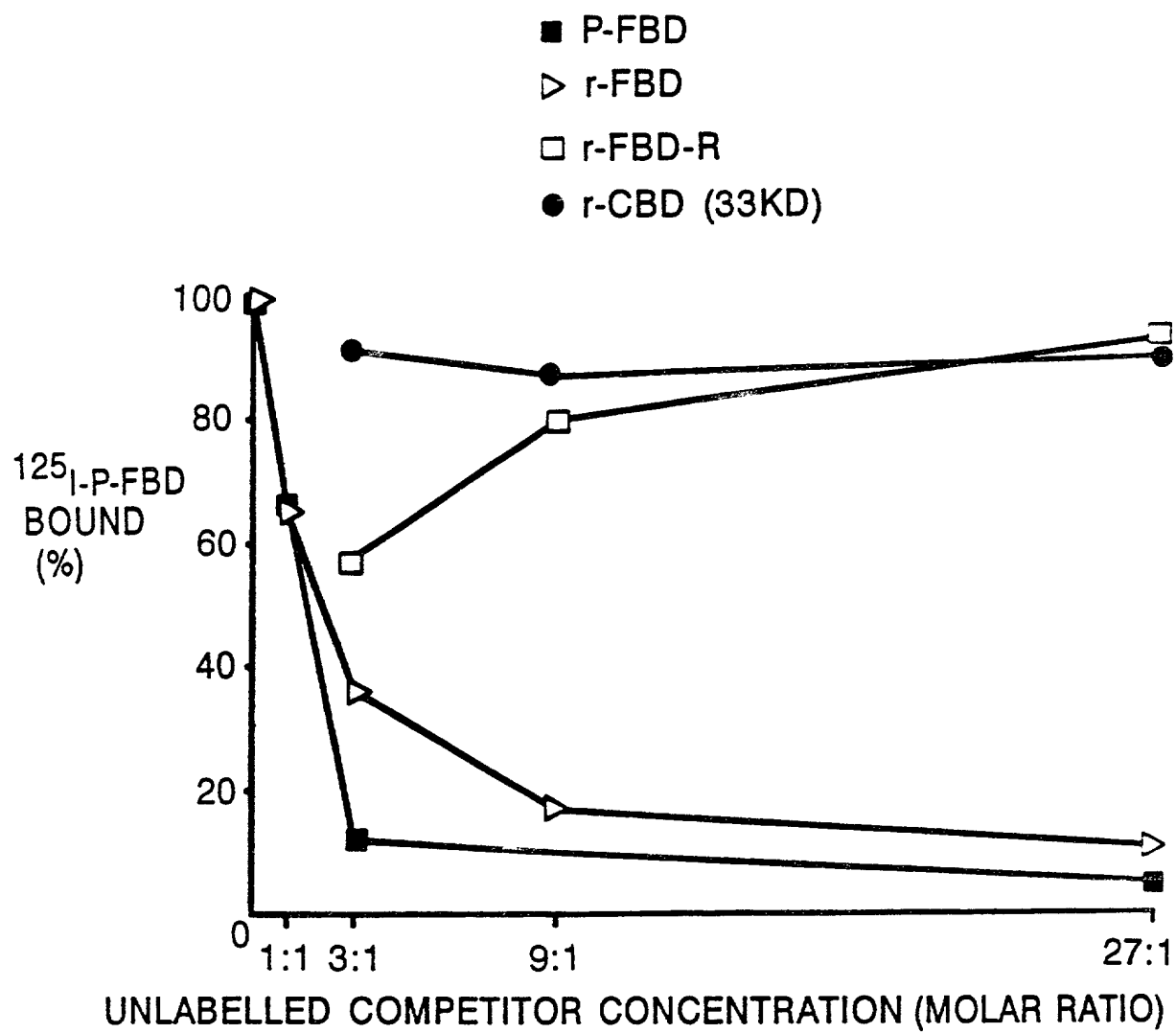
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FIGURE 25



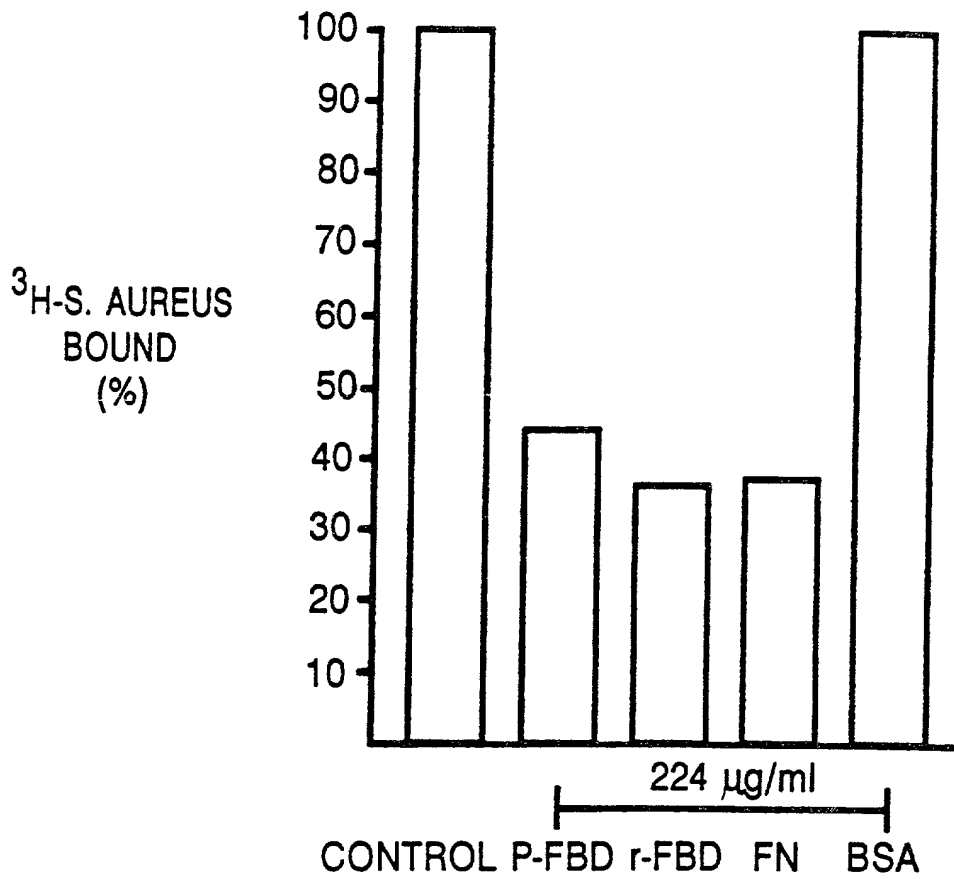
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FIGURE 26



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FIGURE 27



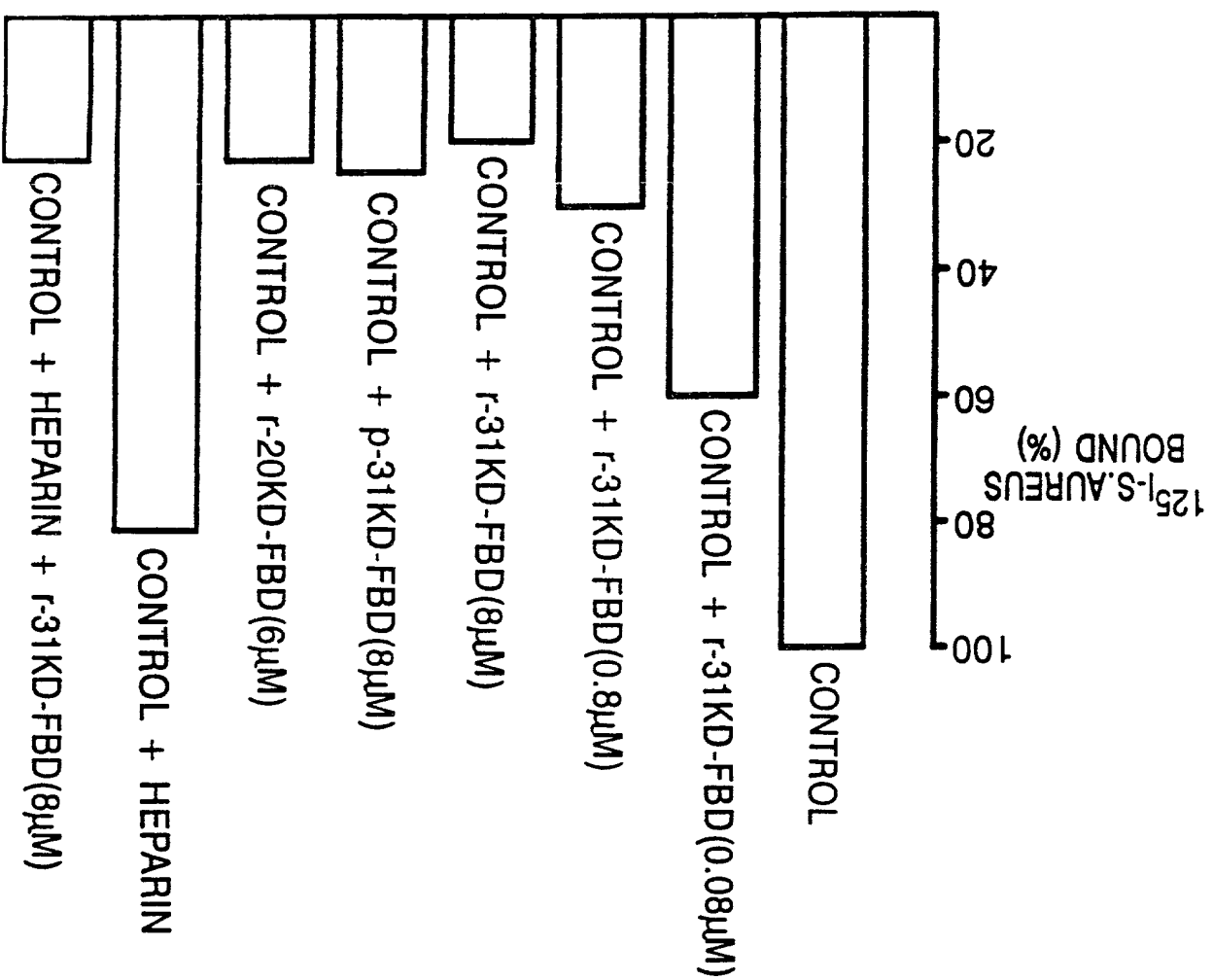


FIGURE 28

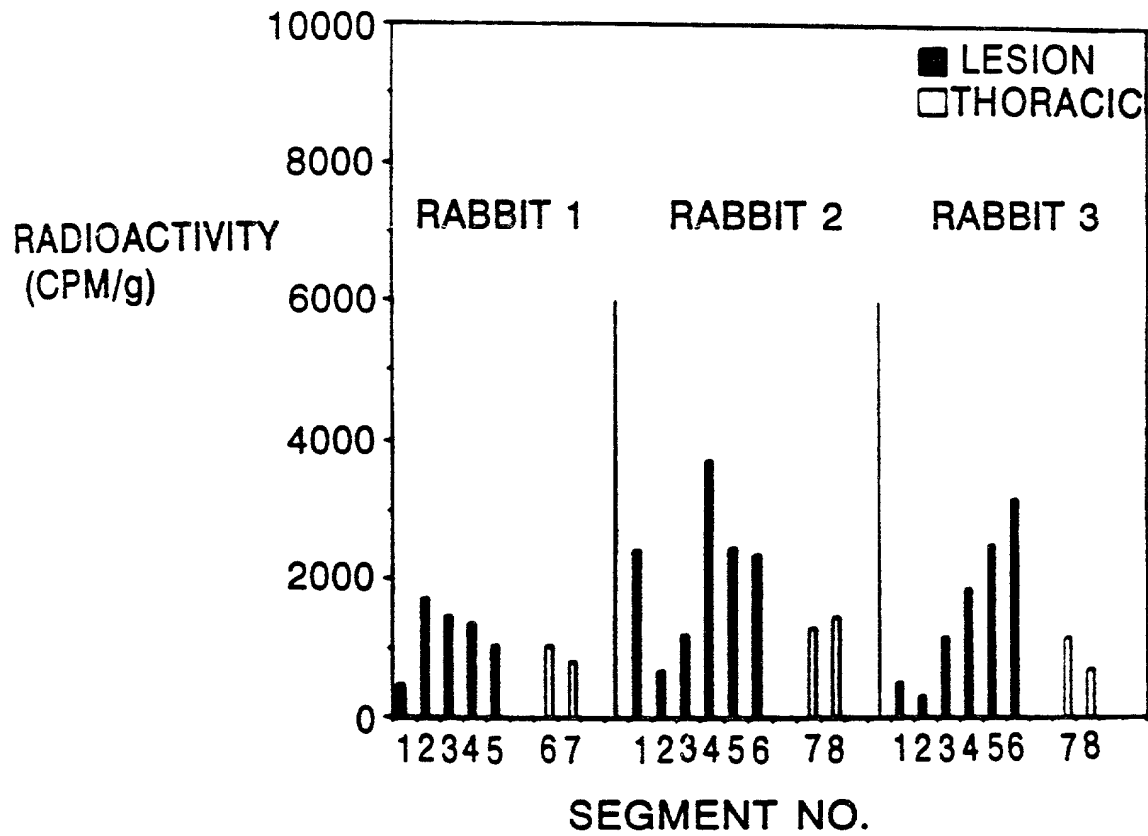
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03432371.042700

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FIGURE 29A

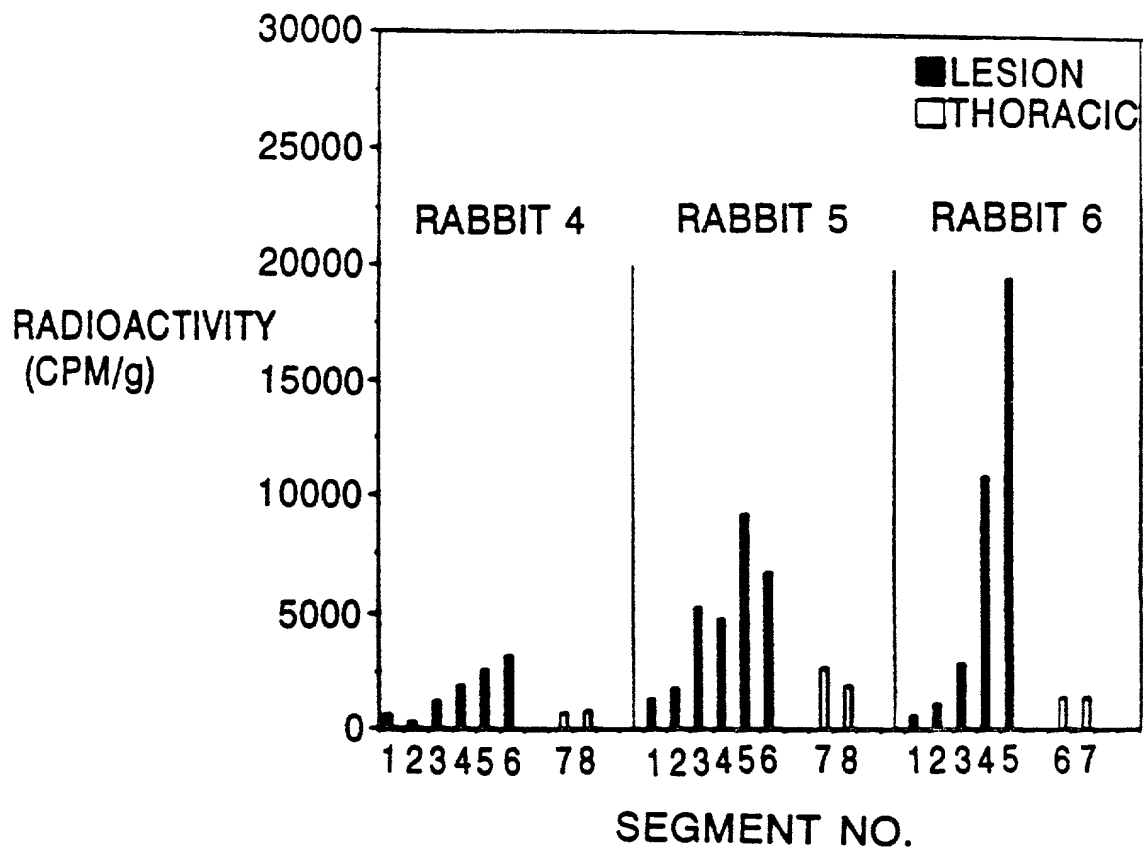
DISTRIBUTION OF RADIOACTIVITY IN RABBIT AORTA
SEGMENTS AFTER FN ADMINISTRATION



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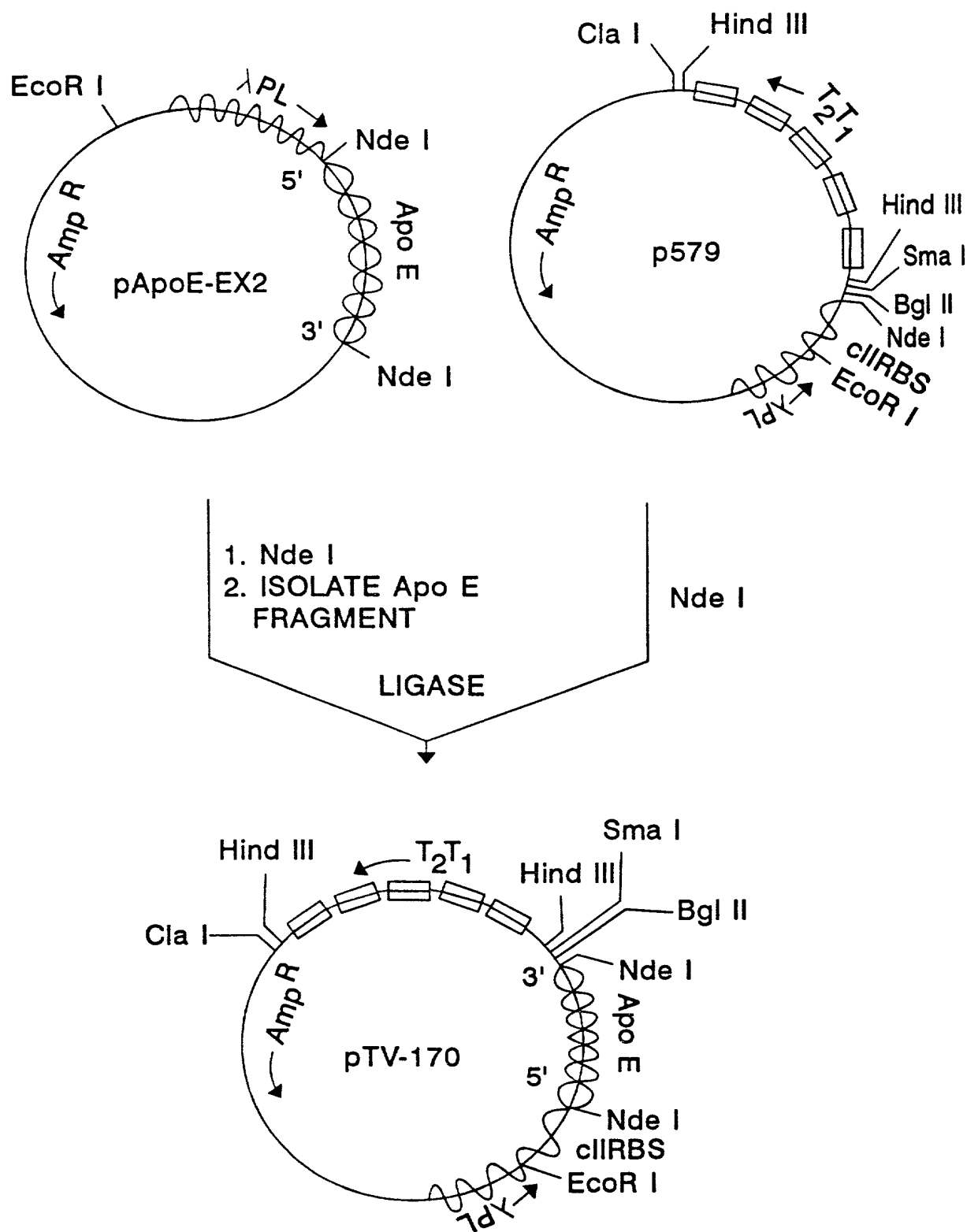
FIGURE 29B

DISTRIBUTION OF RADIOACTIVITY IN RABBIT AORTA
SEGMENTS AFTER 31kD FBD ADMINISTRATION



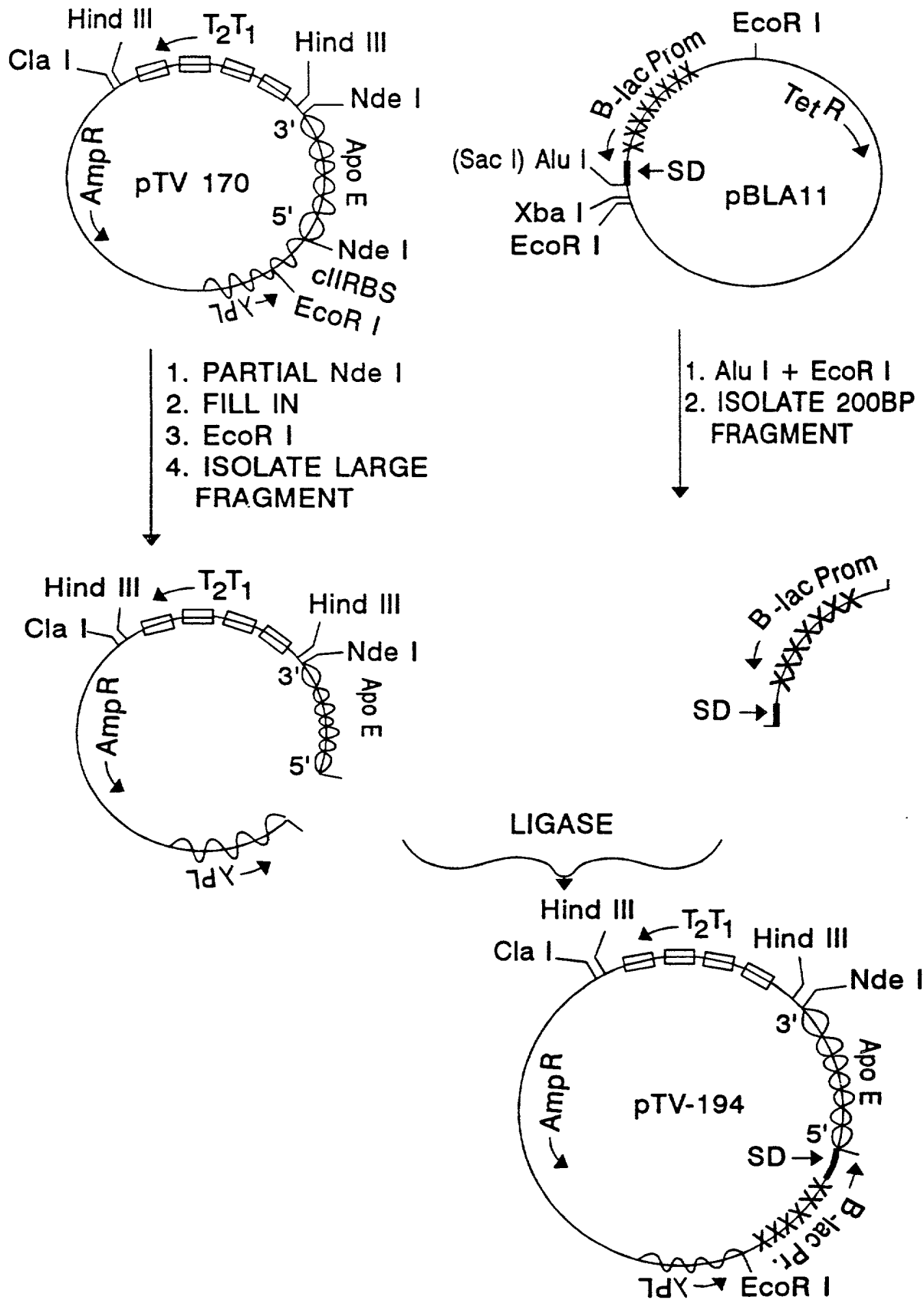
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FIGURE 30



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FIGURE 31



09493971.012700

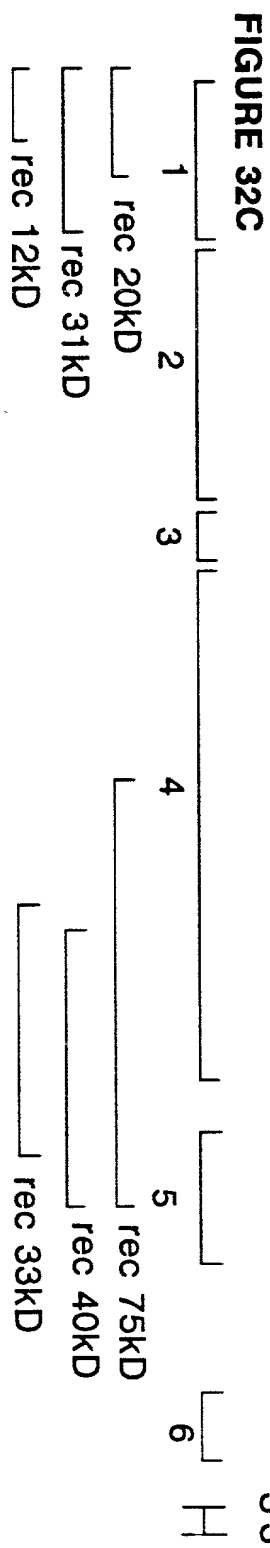
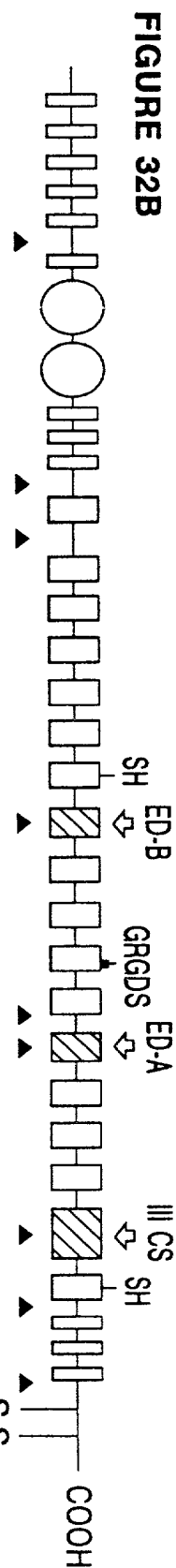
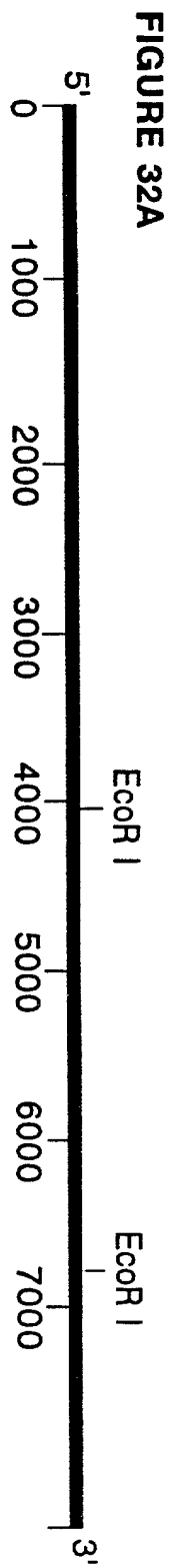


FIGURE 32E

	NUCLEOTIDES	
FBD	rec-12kD:	14-340
	rec-20kD:	14-472
	rec-31kD:	14-799
CBD	rec-40kD:	4151-5566
	rec-75kD:	3317-5566
	rec-33kD:	3998-5179

FIGURE 33

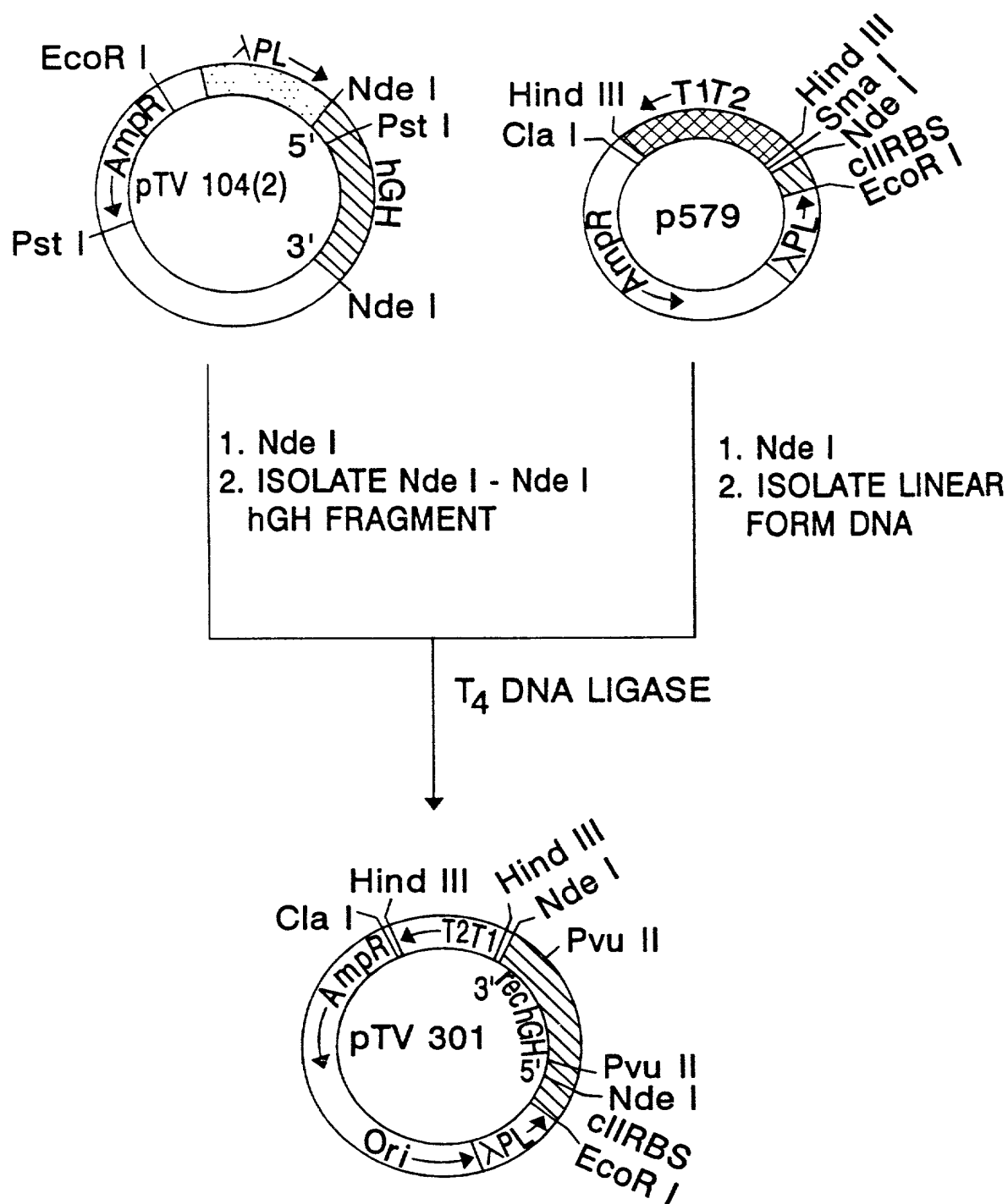


FIGURE 34

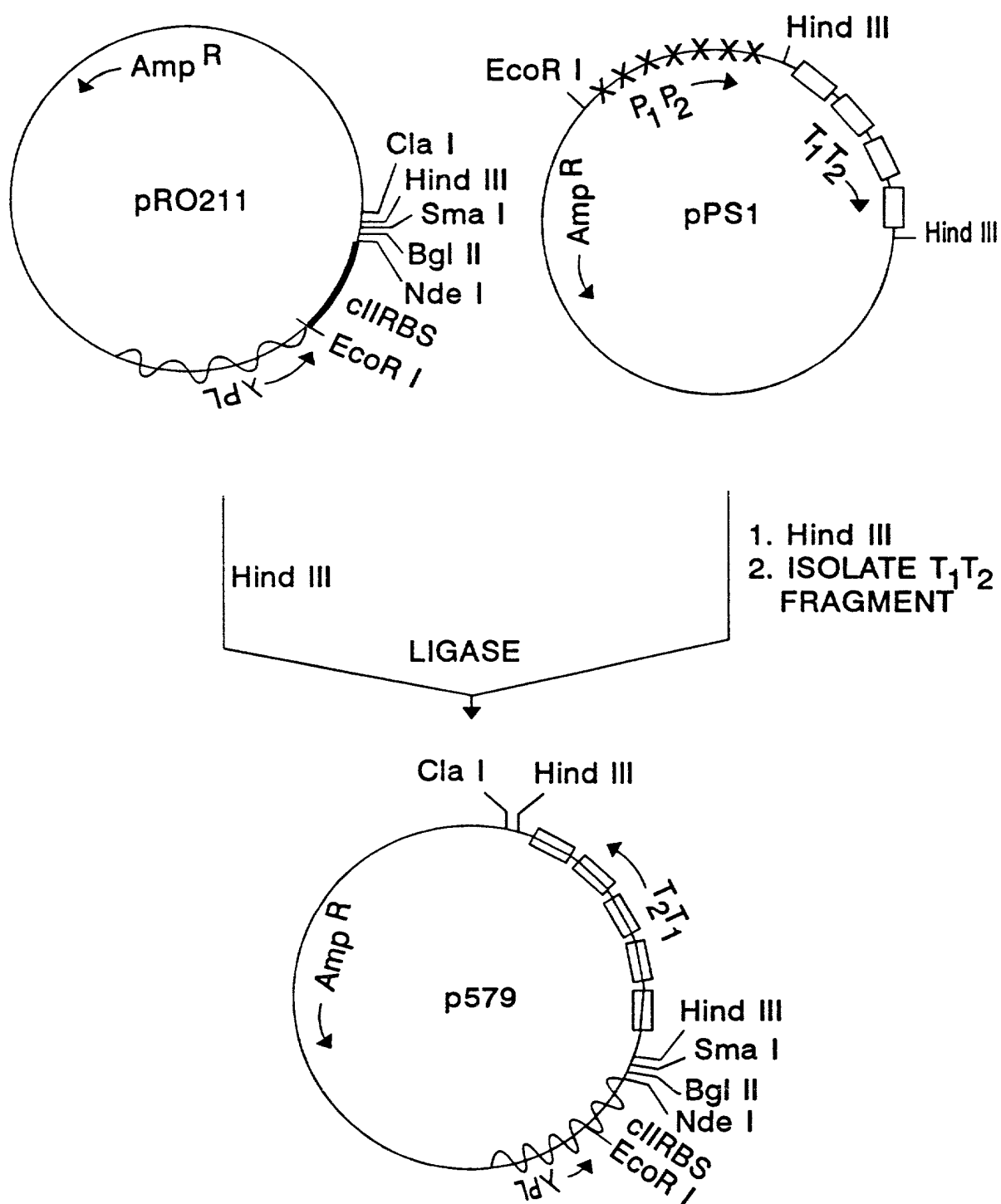
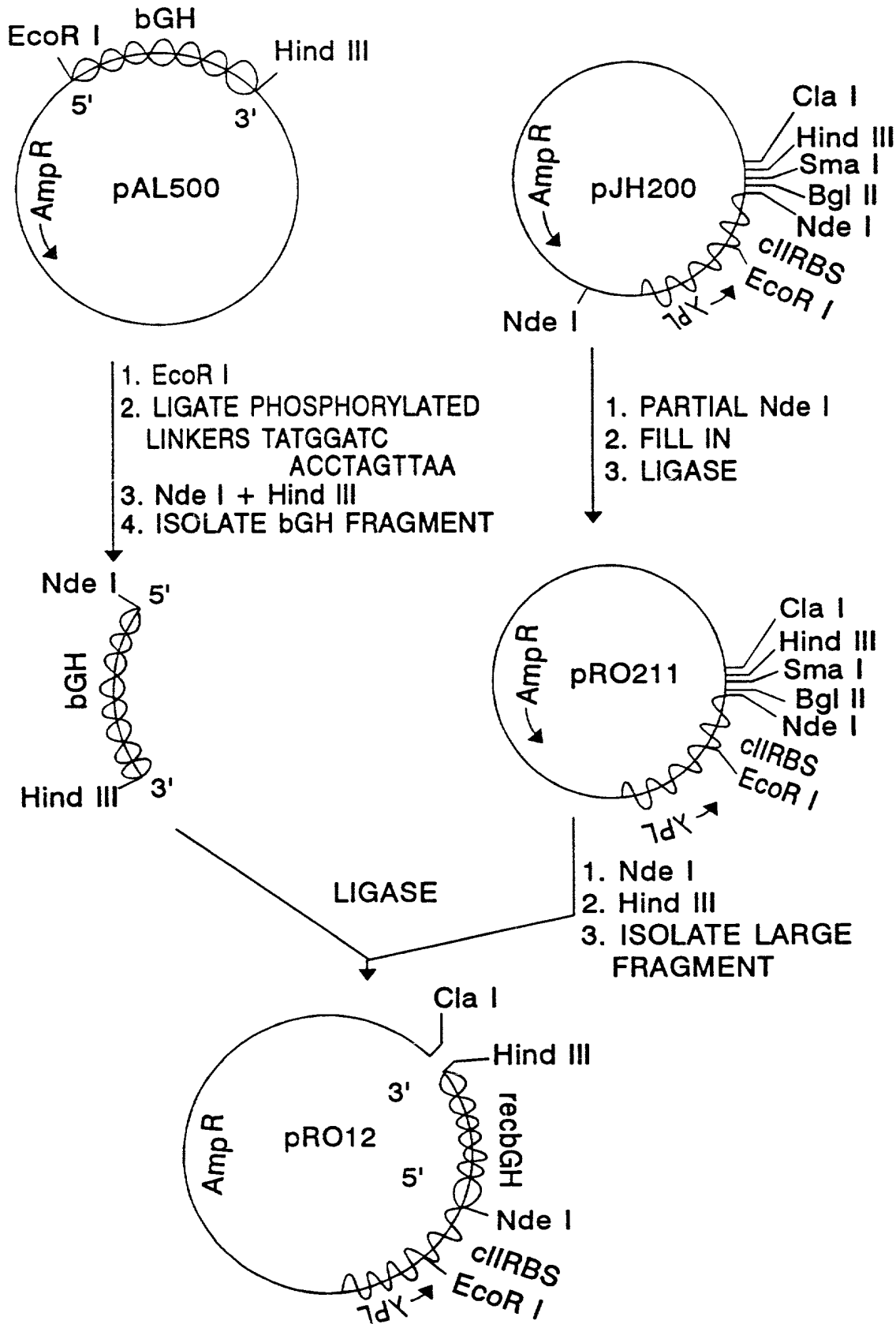
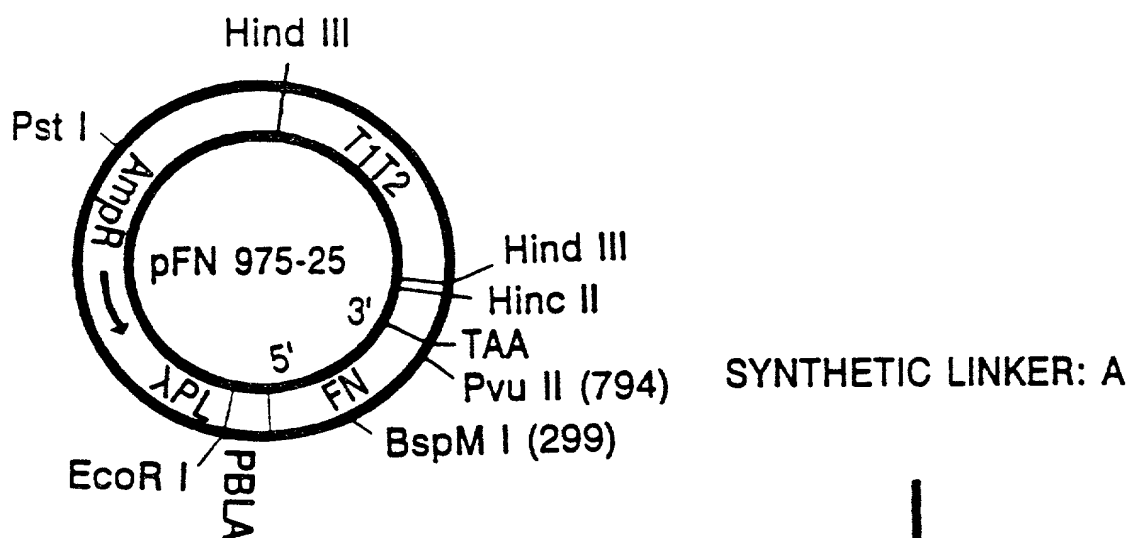


FIGURE 35

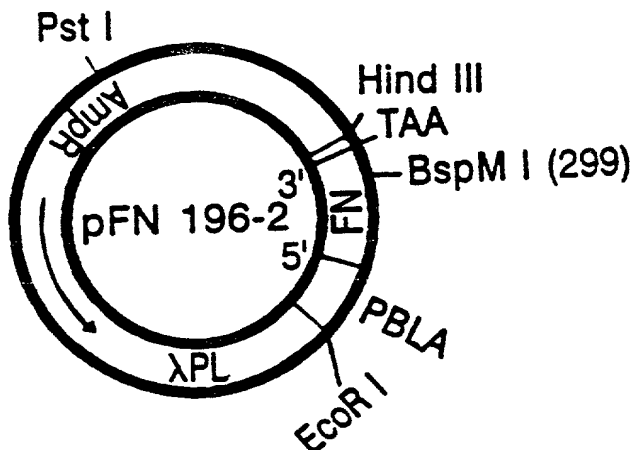


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FIGURE 36



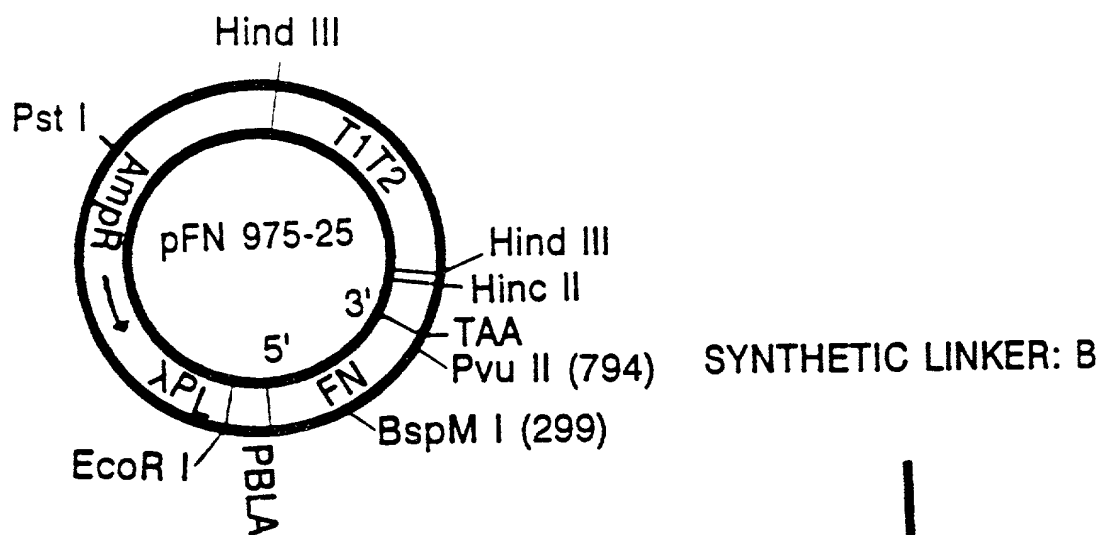
1. DIGEST WITH BspM I + Hind III
2. ISOLATE LARGE BspM I - Hind III FRAGMENT

T₄ DNA LIGASE



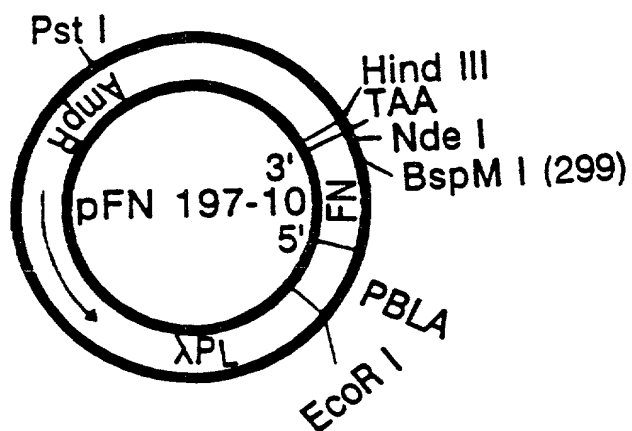
09492971.012700

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FIGURE 37



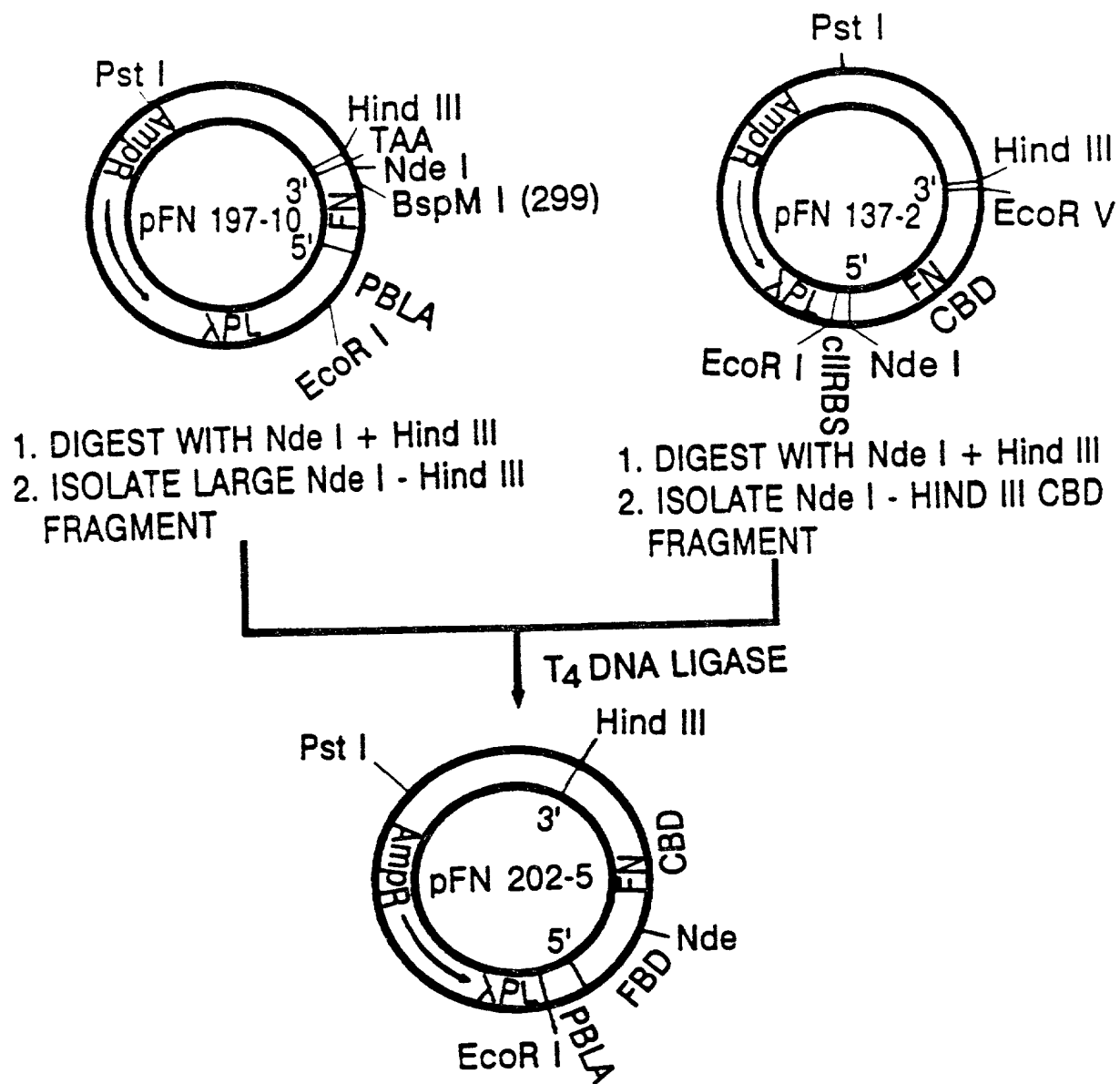
1. DIGEST WITH BspM I + Hind III
2. ISOLATE LARGE BspM I - Hind III FRAGMENT

T₄ DNA LIGASE



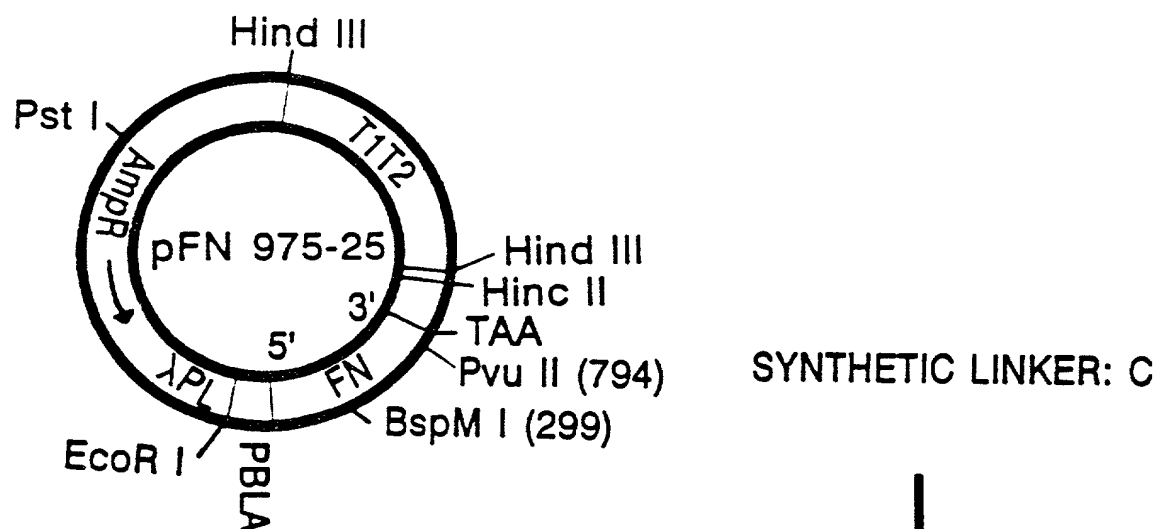
09492971.012700

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FIGURE 38

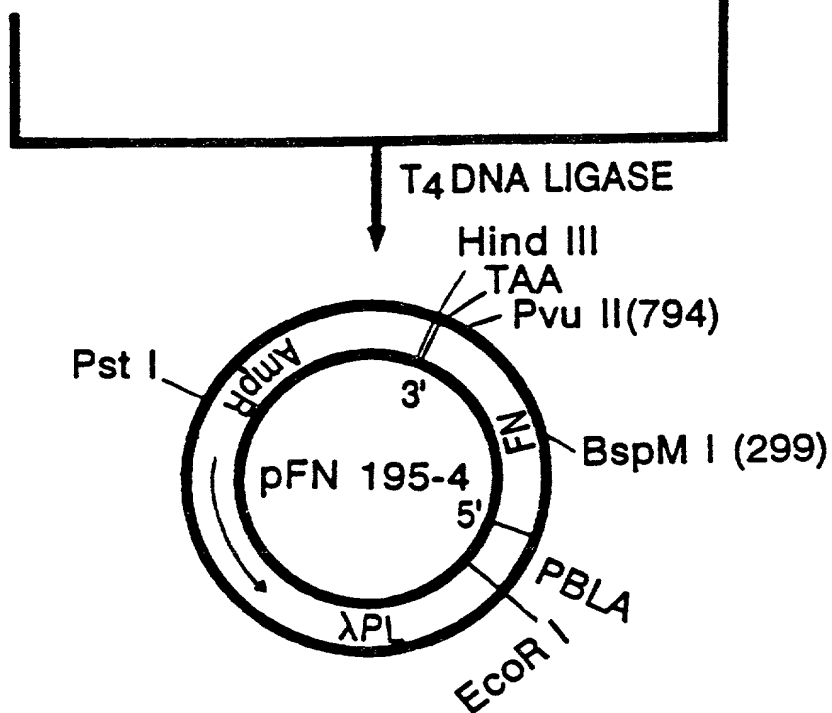


00443971.013700

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FIGURE 39

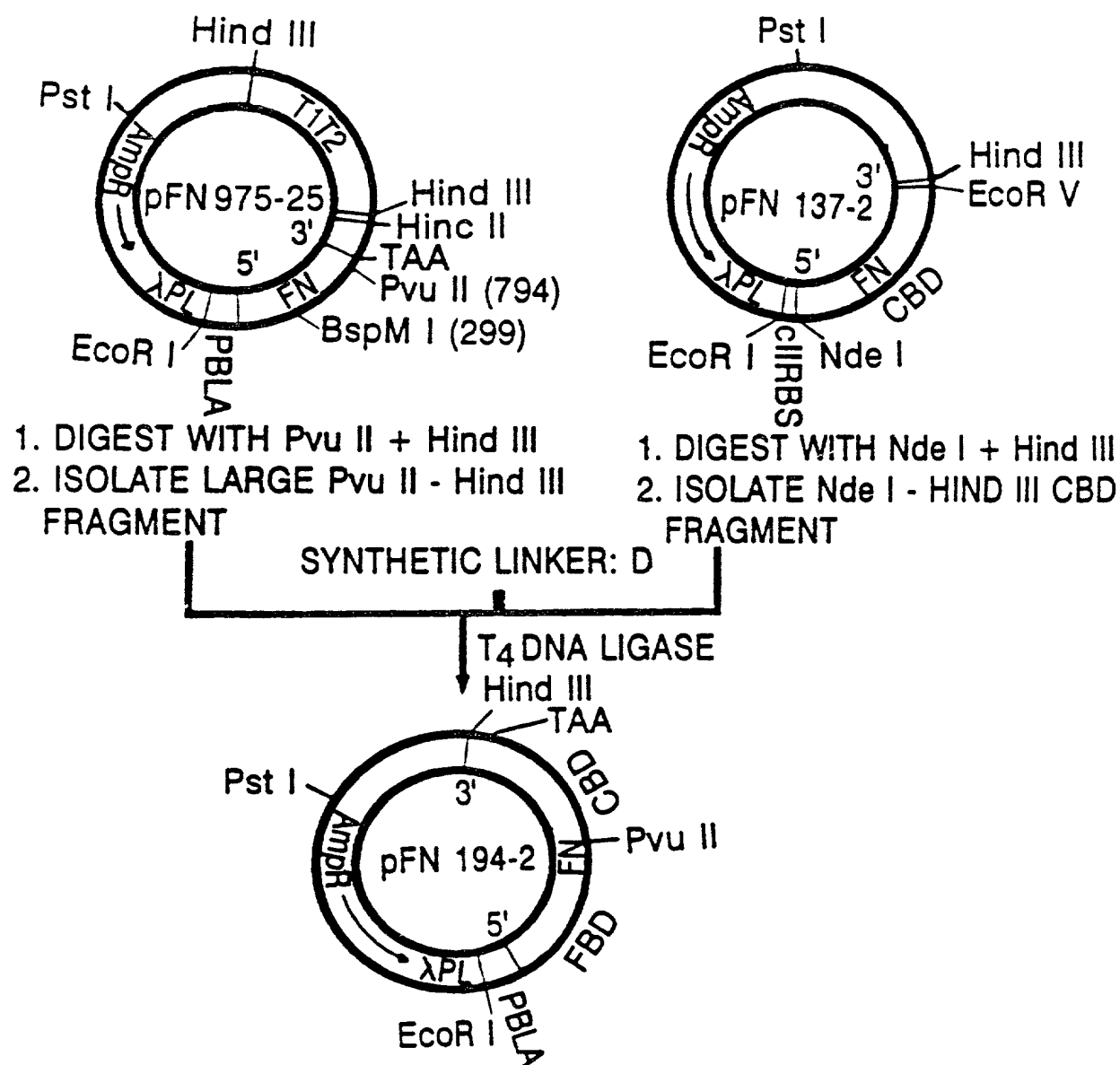


1. DIGEST WITH Pvu II + Hind III
2. ISOLATE LARGE Pvu II - Hind III FRAGMENT



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FIGURE 40



0044926971.012700

FIGURE 41

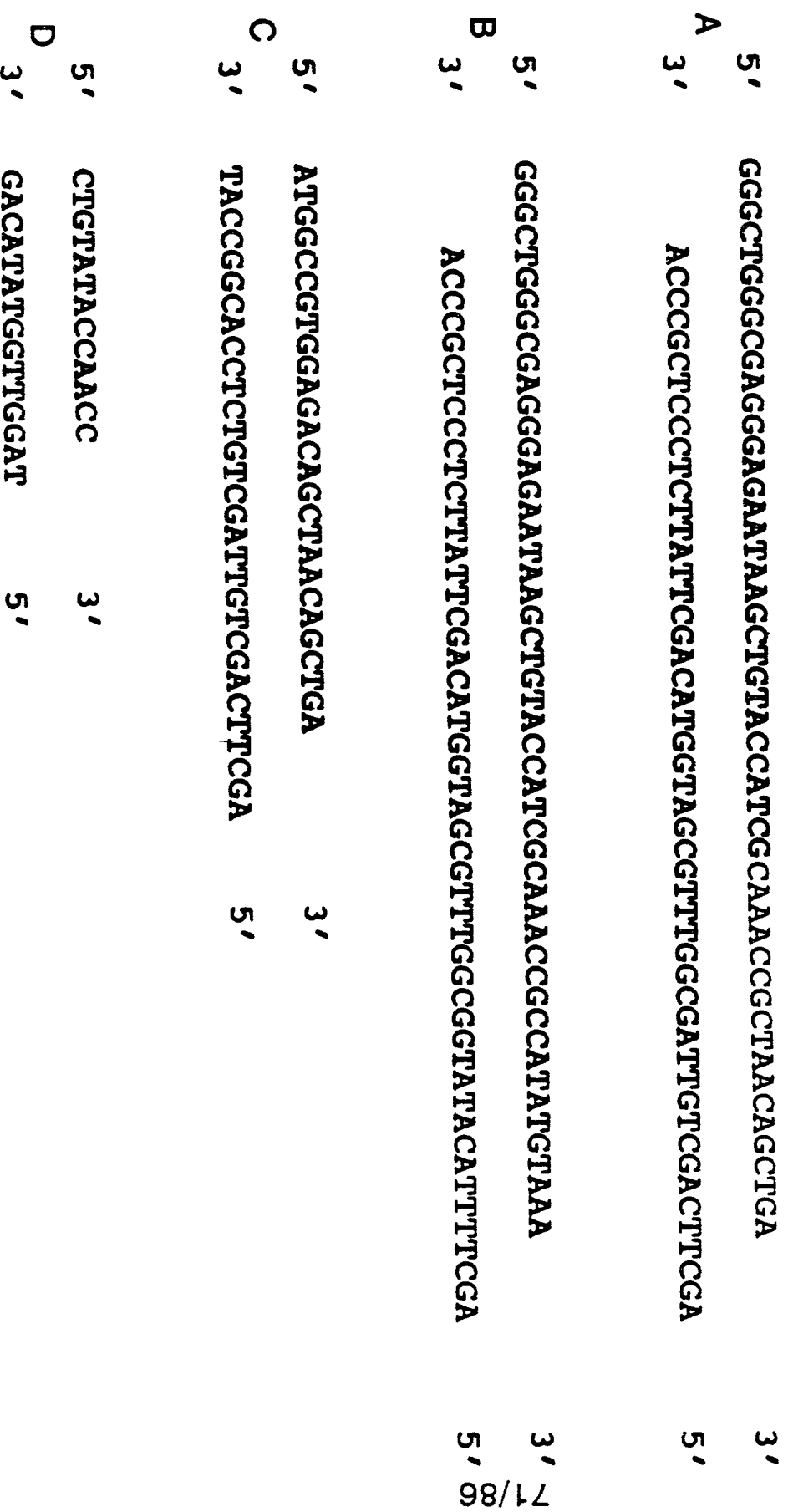


FIGURE 42

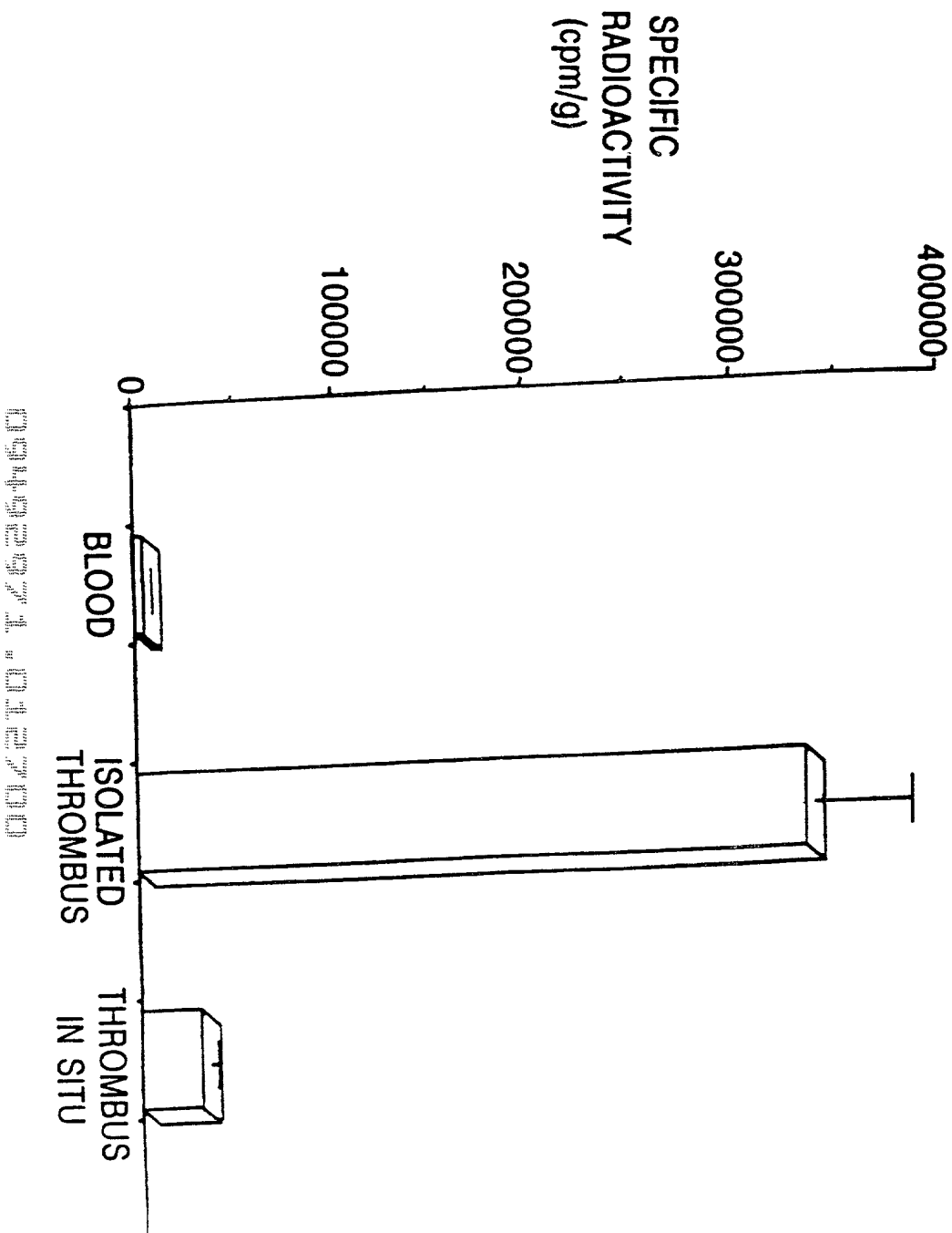
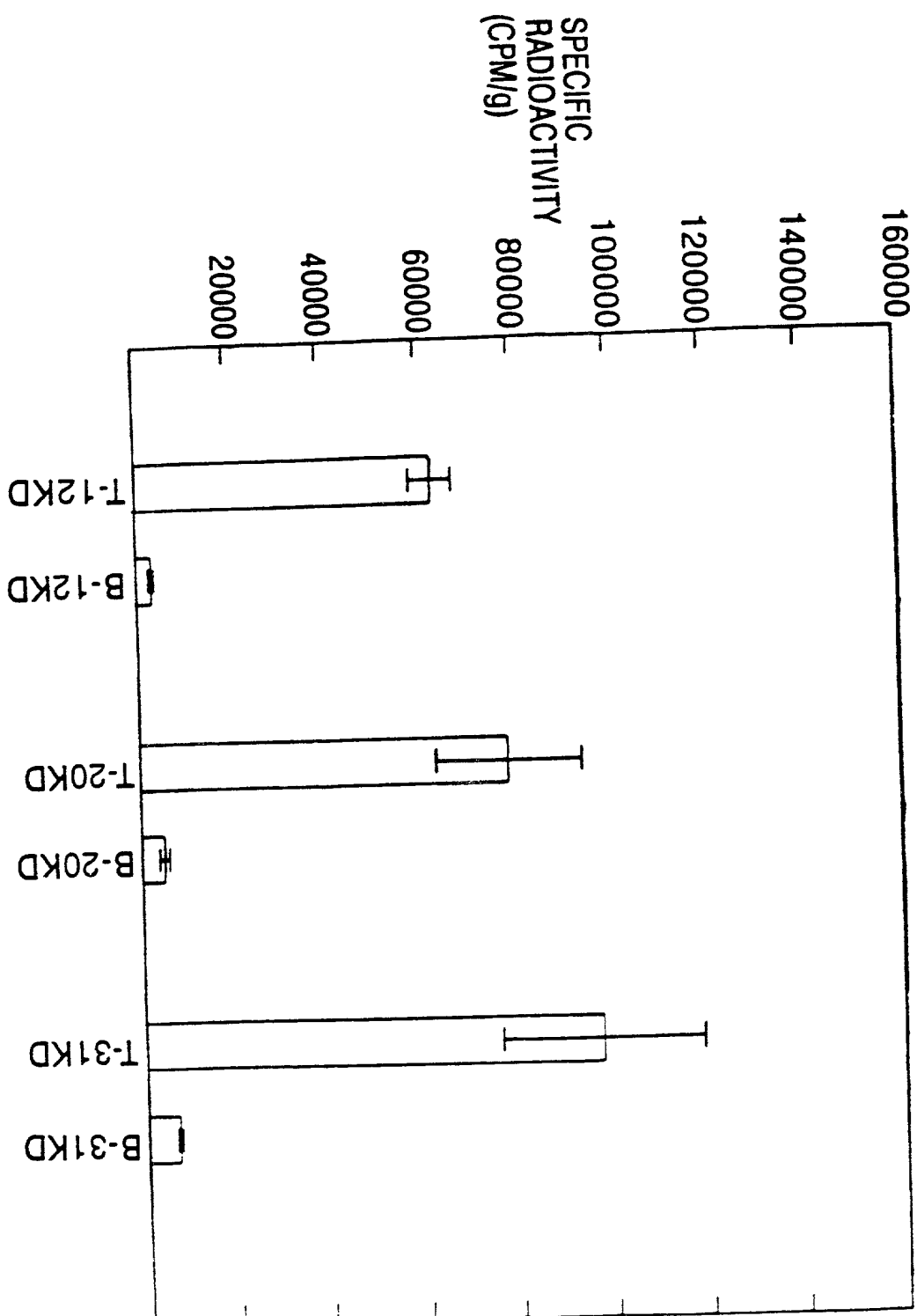


FIGURE 43

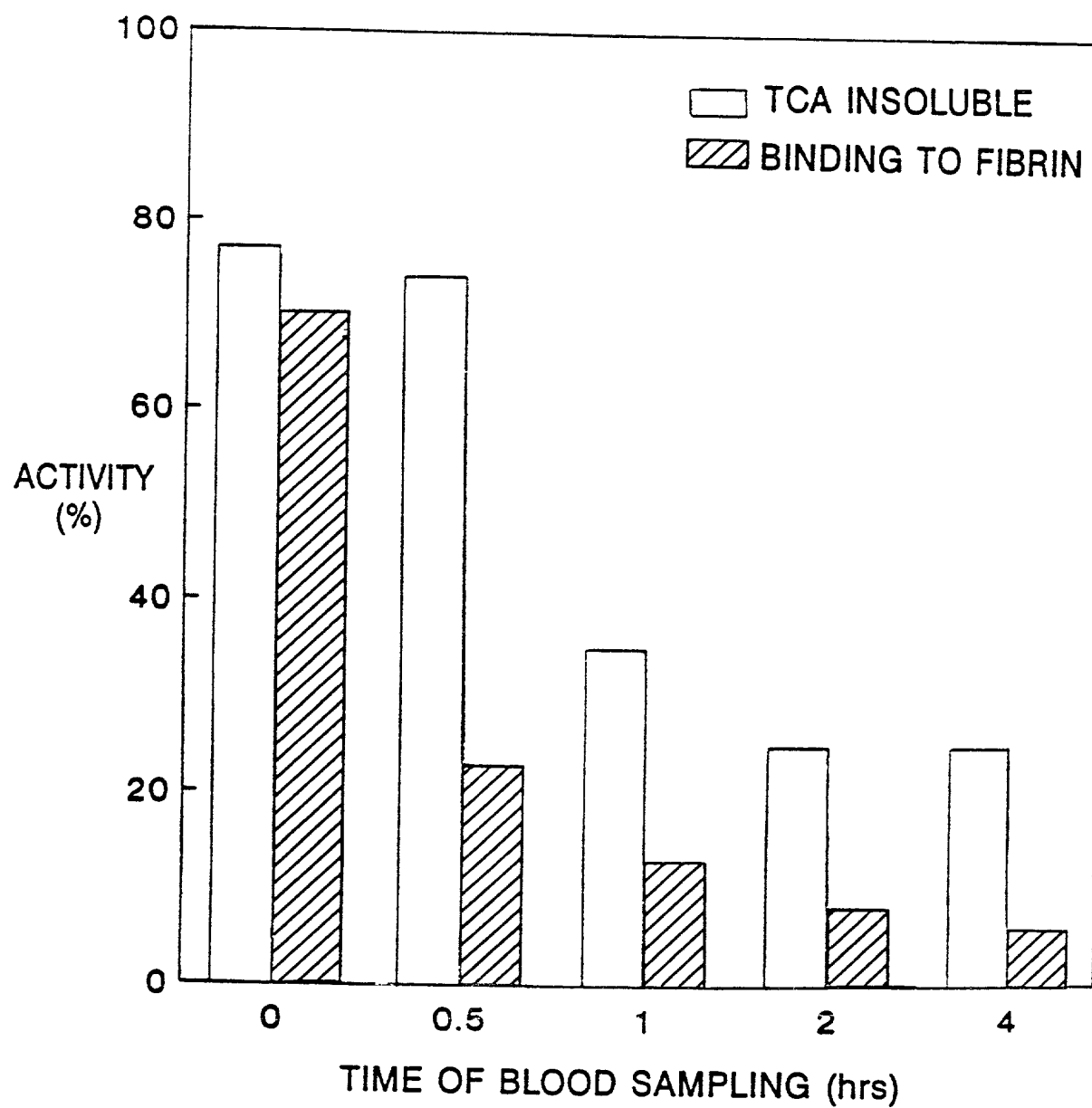


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FIGURE 44



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FIGURE 45

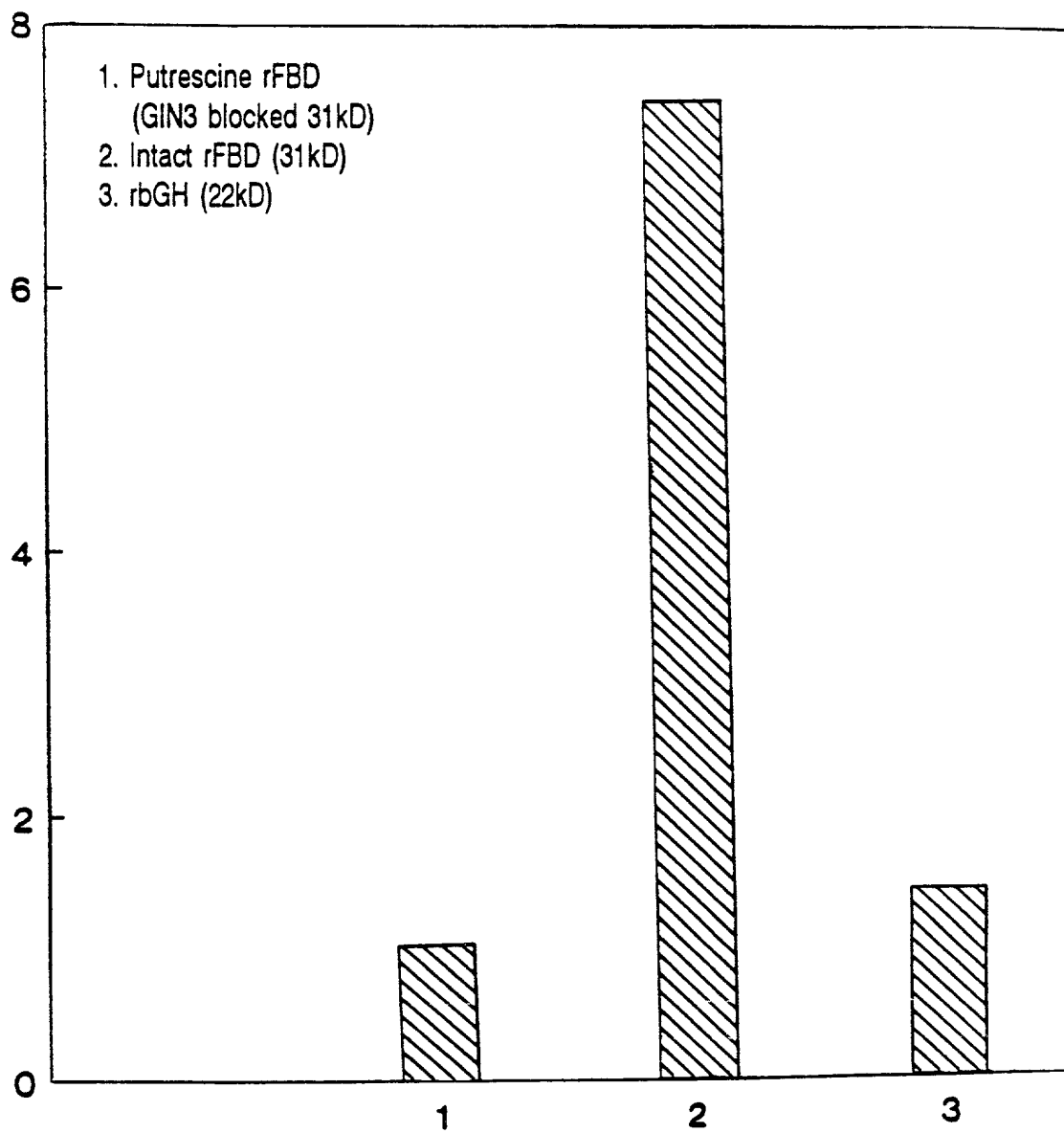


FIGURE 46A

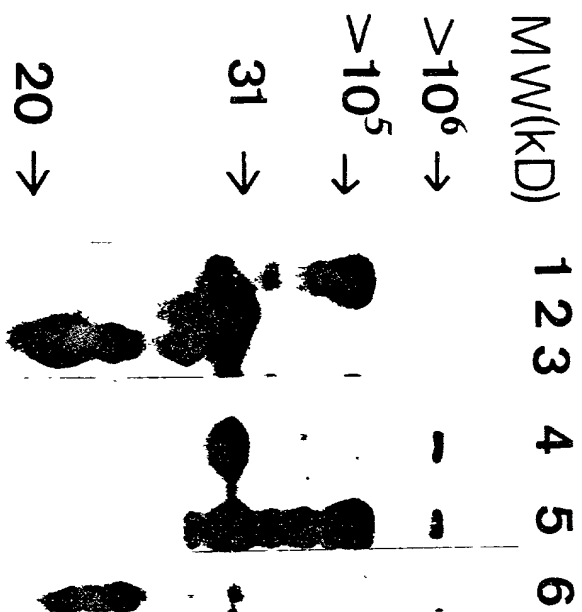


FIGURE 46B

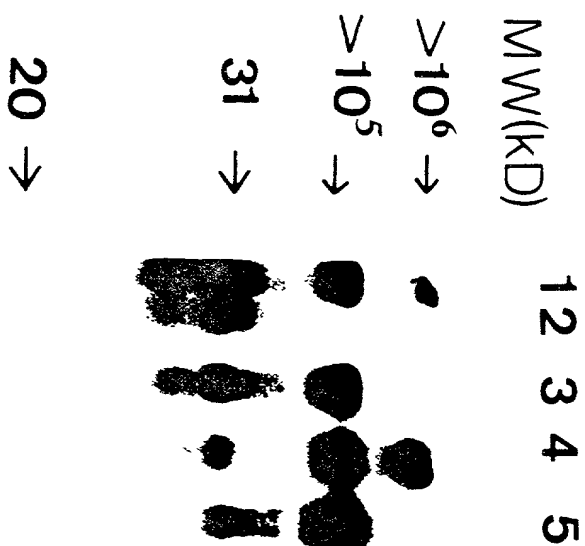


FIGURE 47A

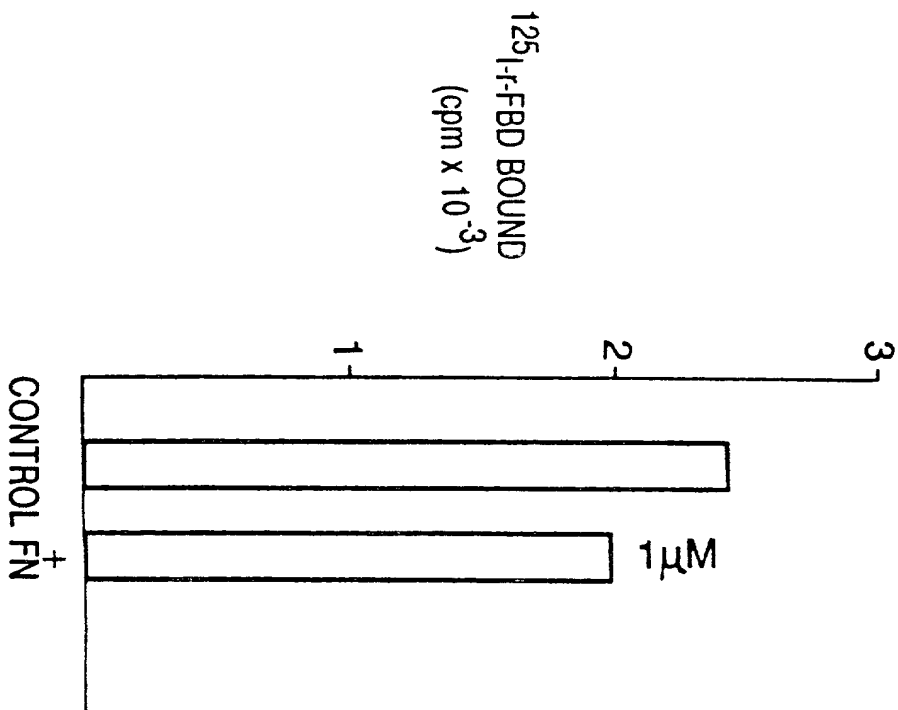
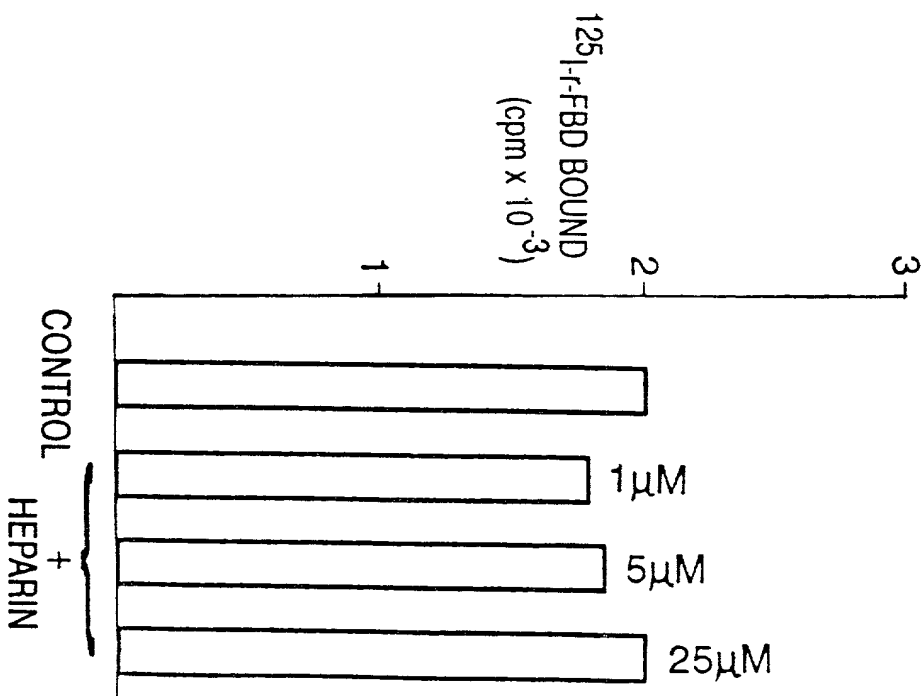
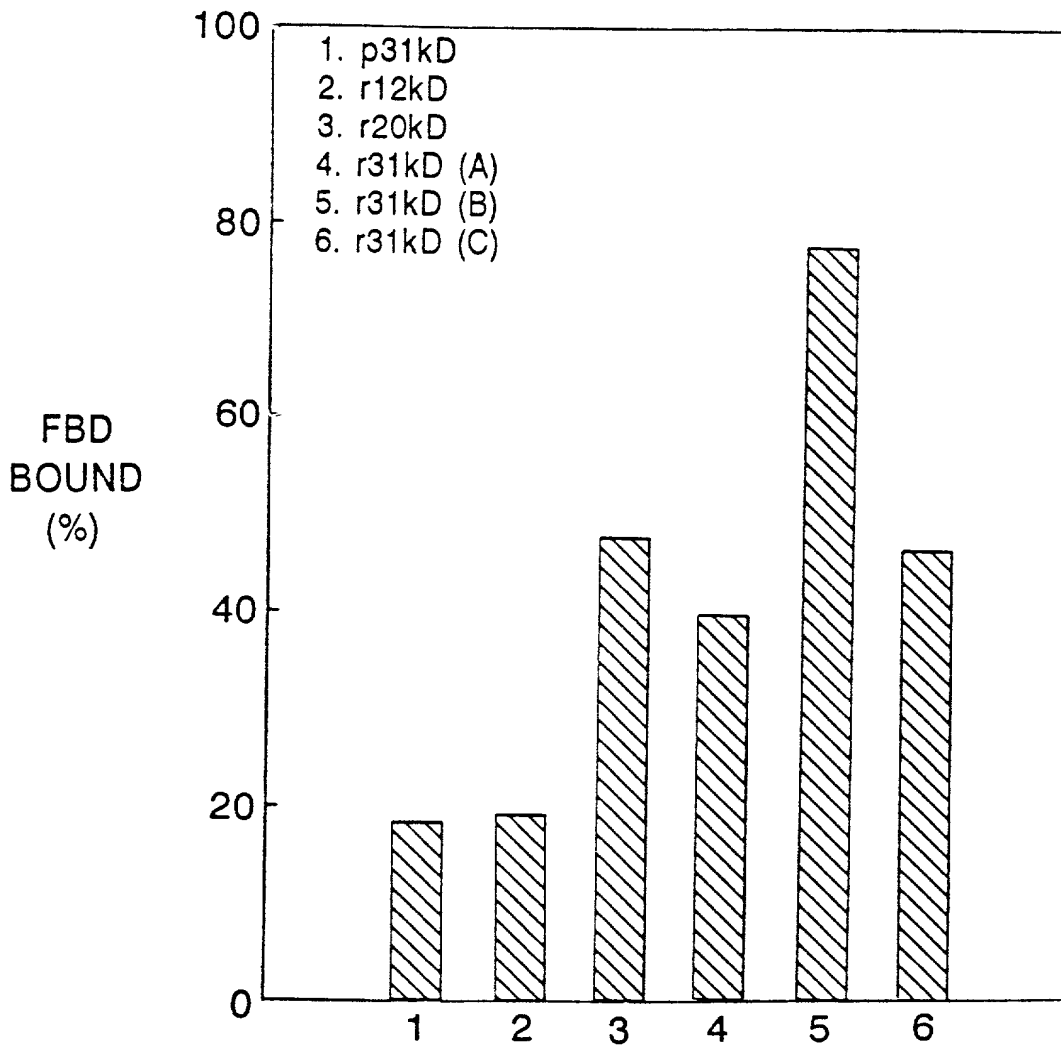


FIGURE 47B



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FIGURE 48



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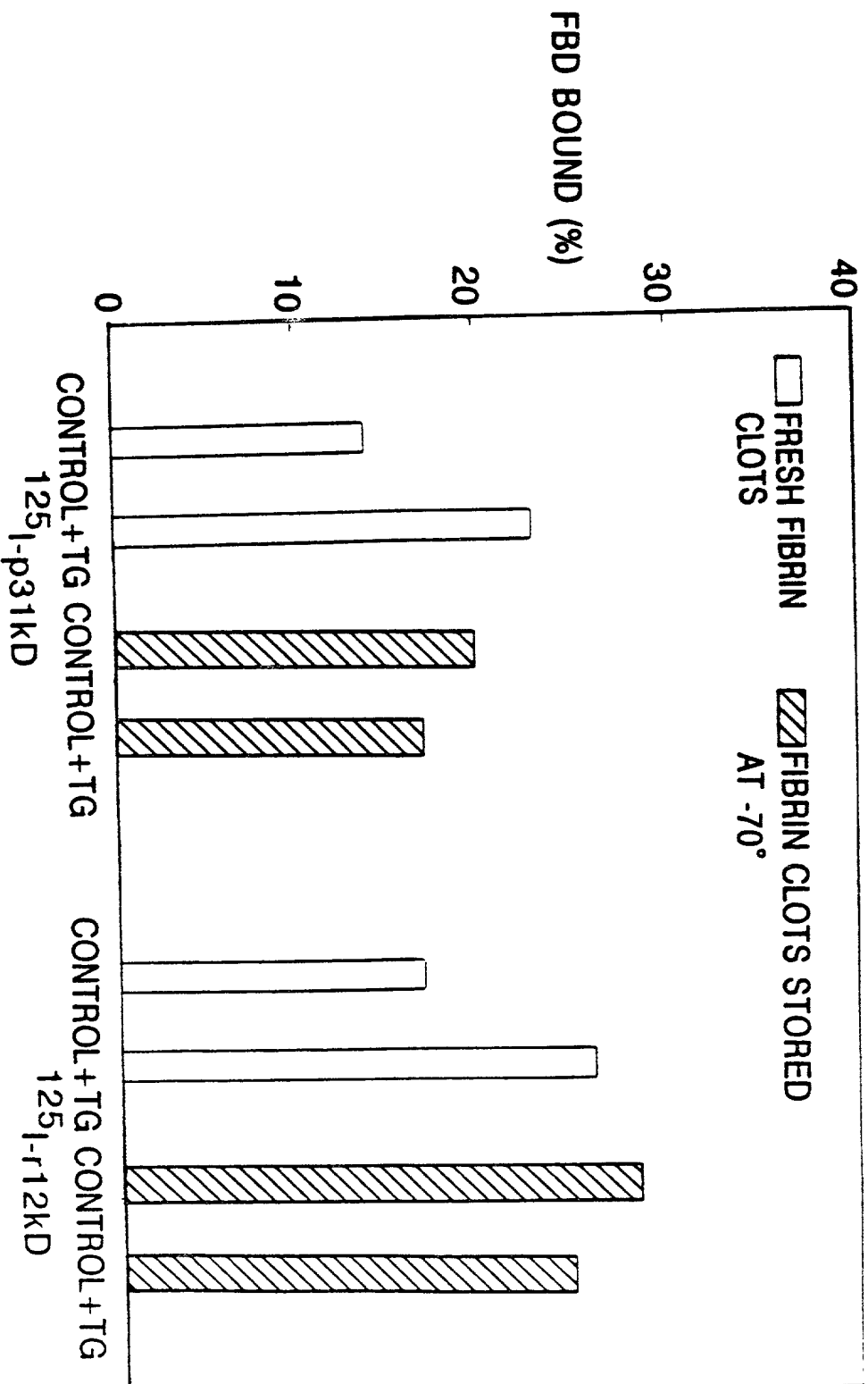


FIGURE 50A

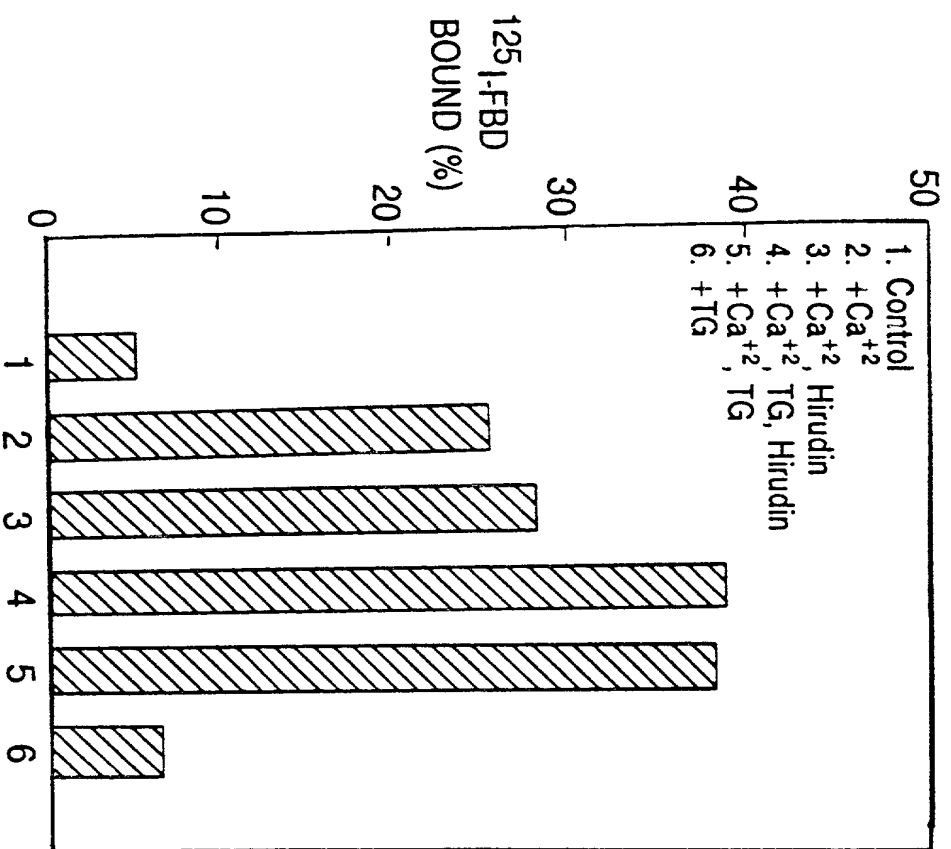
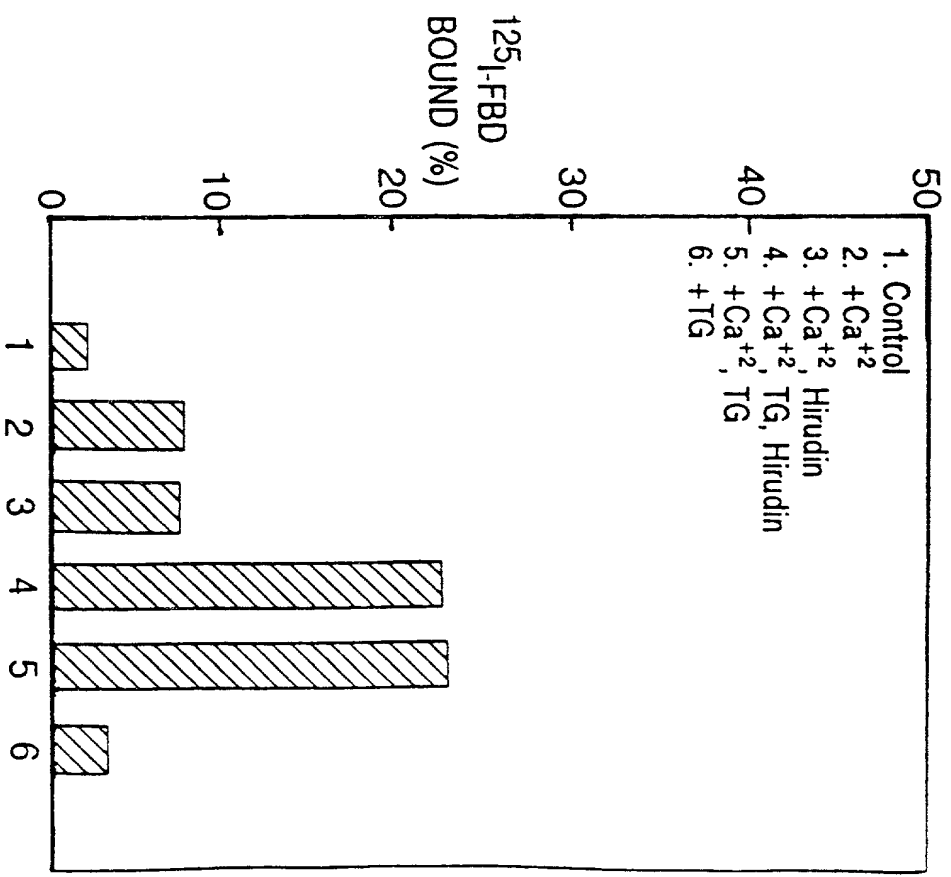


FIGURE 50B



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09492971.012700

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FIGURE 51

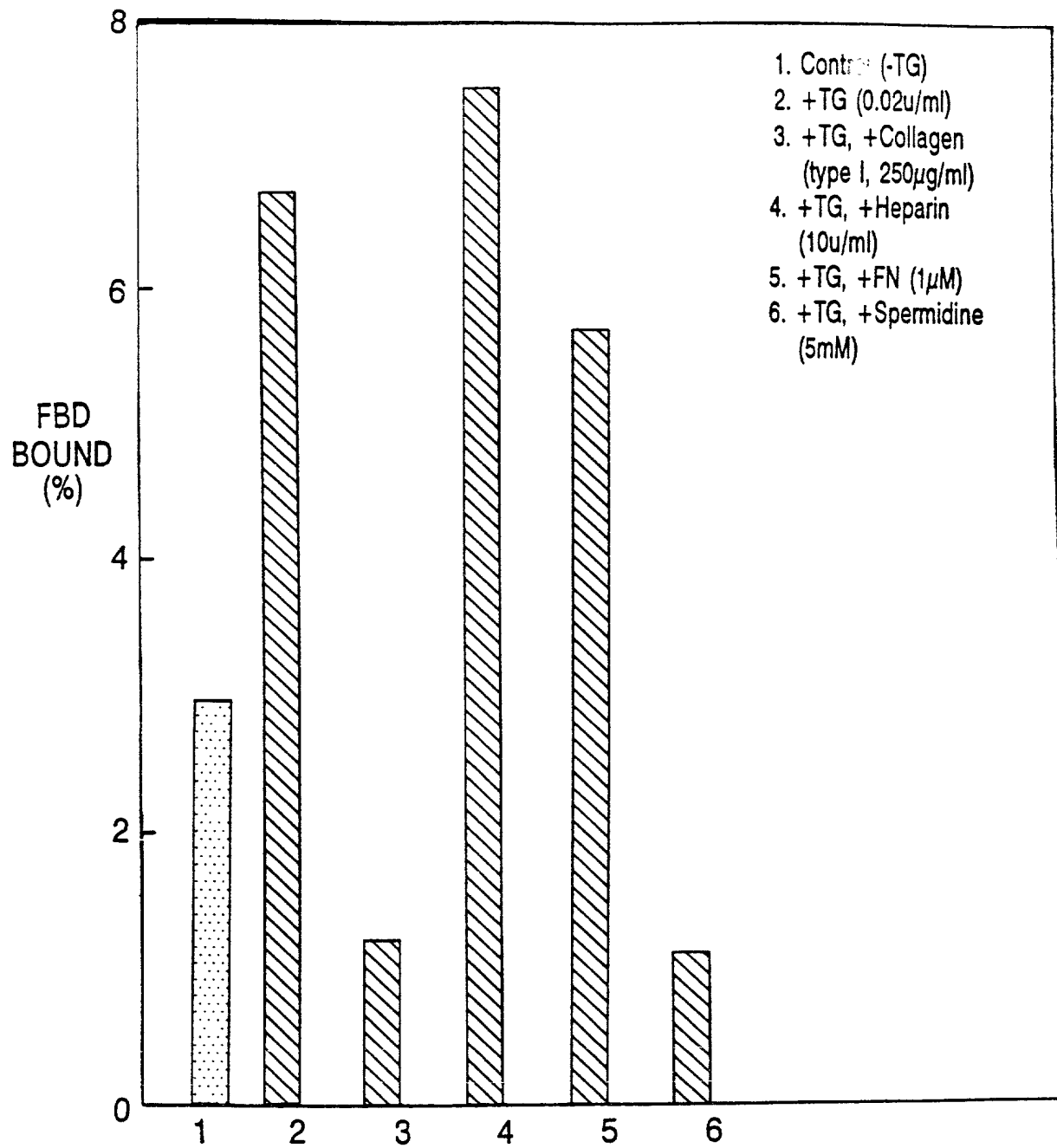


FIGURE 52A-1

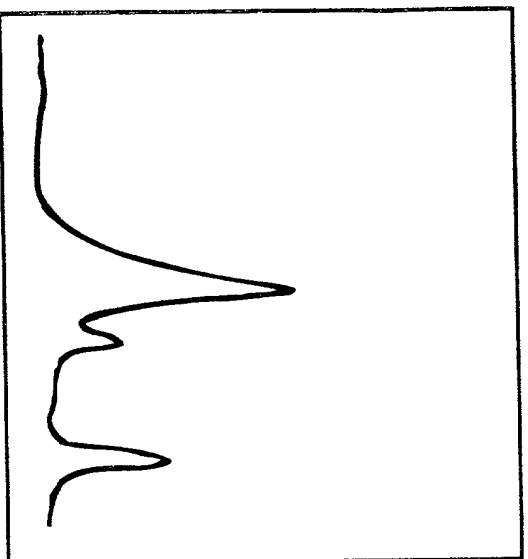


FIGURE 52A-2

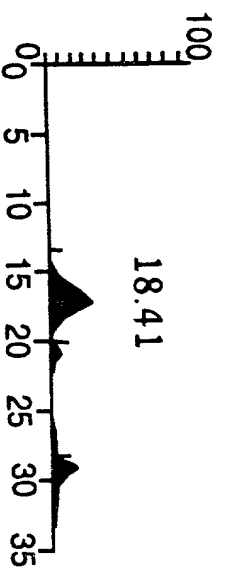


FIGURE 52B-1

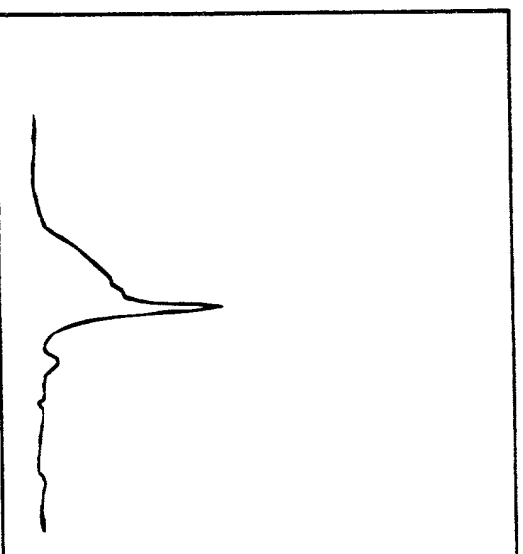


FIGURE 52B-2

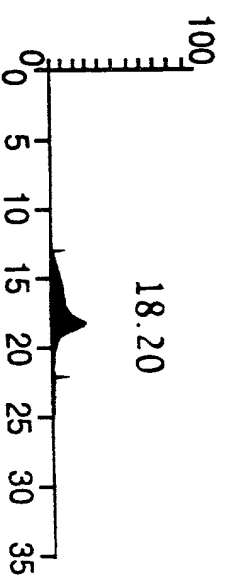


FIGURE 52C-1

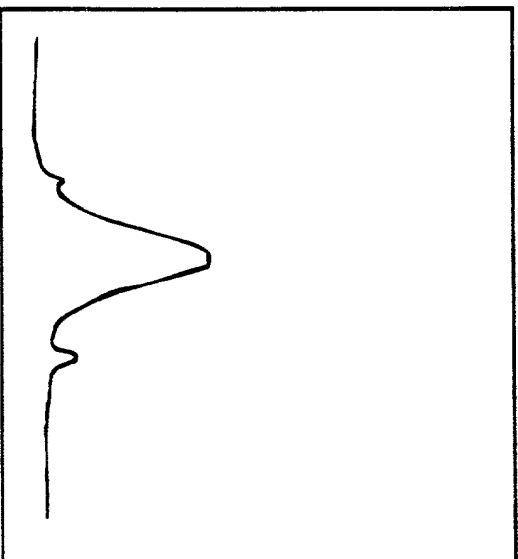


FIGURE 52C-2

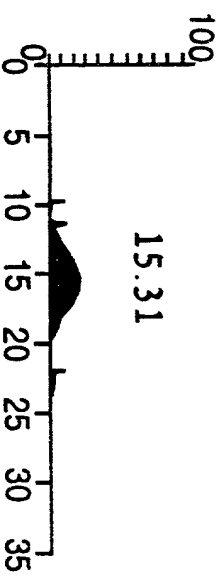


FIGURE 52D-2

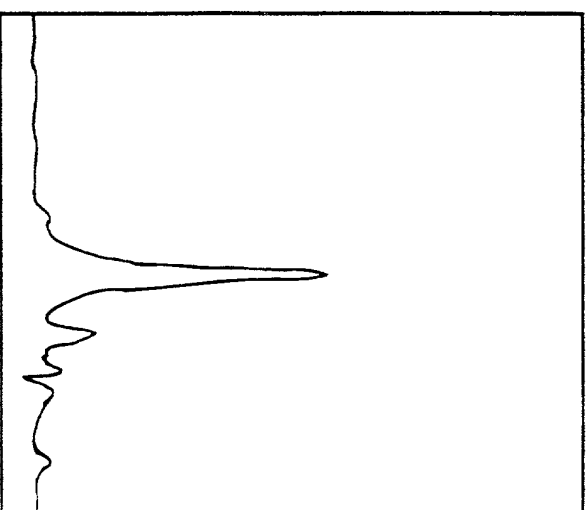


FIGURE 52D-1

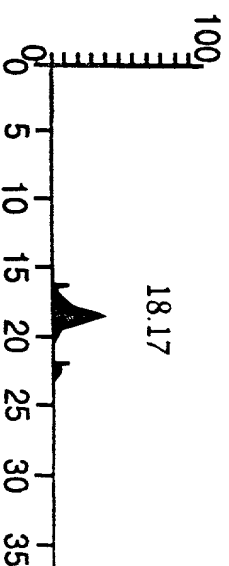


FIGURE 52E-1

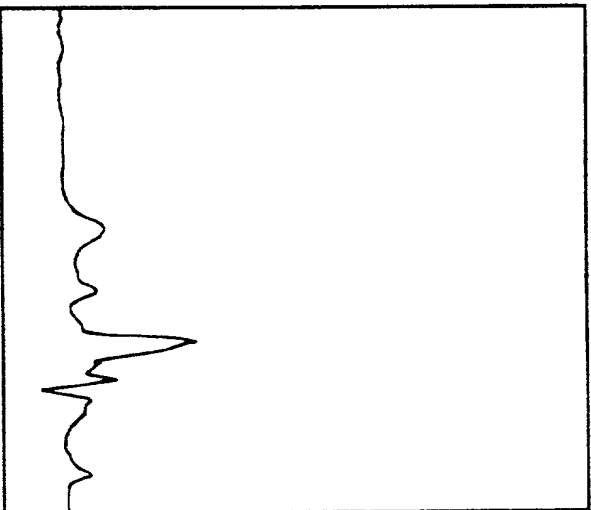


FIGURE 52E-2

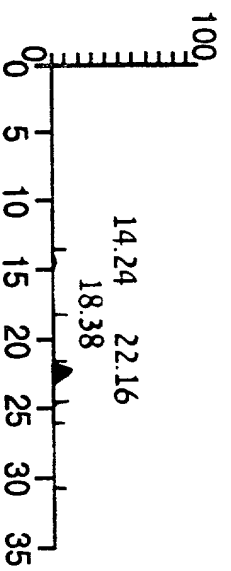


FIGURE 52F-1

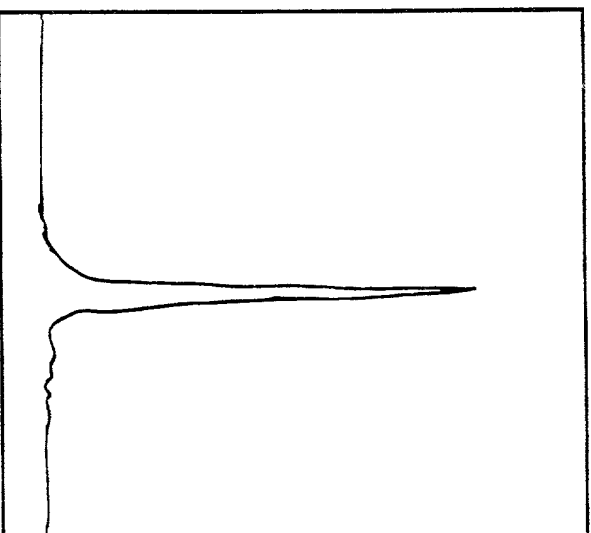


FIGURE 52F-2

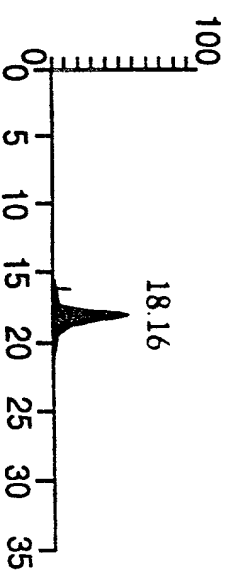


FIGURE 53A

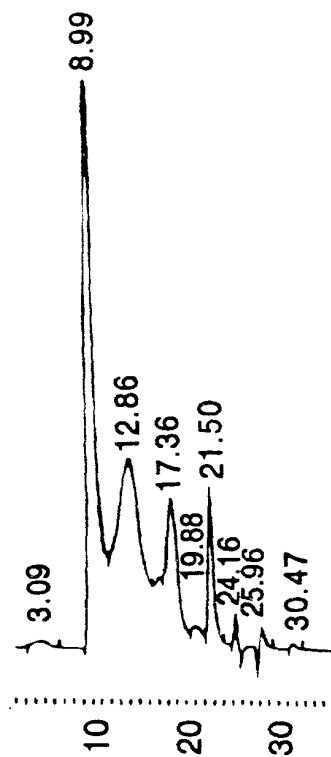


FIGURE 53B

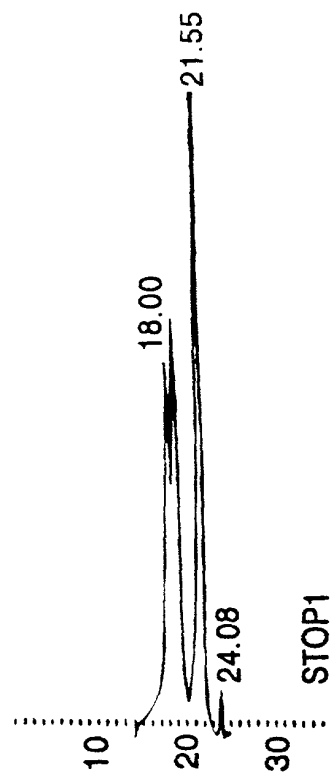


FIGURE 53C

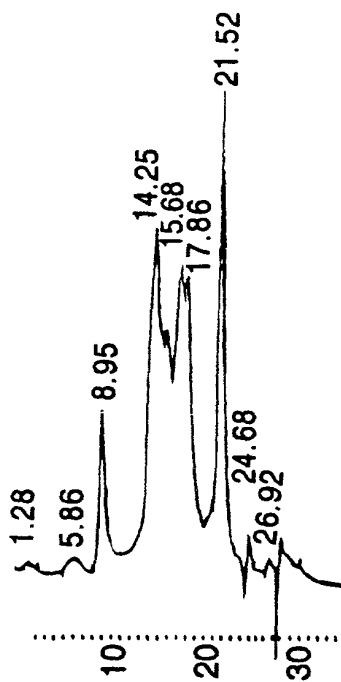


FIGURE 53D

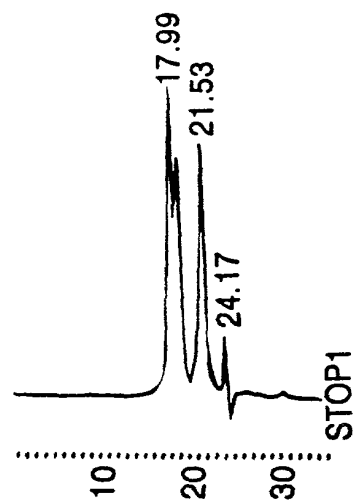


FIGURE 53E

